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Ana Manuela Onofre Meireles

FLAVONOID BIOACTIVITY: TRANSPORT ACROSS BLOOD-BRAIN BARRIER AND NEUROPROTECTIVE EFFECTS. IN VIVO STUDIES.

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR EM METABOLISMO - CLÍNICA E EXPERIMENTAÇÃO, APRESENTADA À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

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Doutor Mário José Cerqueira Gomes Braga

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CONTRIBUTO PESSOAL NOS TRABALHOS DE INVESTIGAÇÃO APRESENTADOS NESTA DISSERTAÇÃO

A candidata declara que teve uma contribuição determinante em toda a realização do trabalho experimental (programação e execução das experiências), bem como na interpretação e discussão dos resultados apresentados em todos os artigos desta dissertação. A candidata contribuiu ainda ativamente na redação dos trabalhos apresentados.

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ABSTRACT

Flavonoids have been pointed as promisor compounds in the prevention of cognitive decline, characteristic of ageing and neurodegenerative diseases. Being synthetized by secondary metabolism of plants, flavonoids are present in a variety of foods and beverages of plant origin such as fruits, vegetables, tea, cocoa and wine. Epidemiological studies have associated the consumption of flavonoid rich foods with lower risk of cognitive decline. Some mechanisms of action of flavonoids had been already unveiled but several others remain to be explored. The present work aimed to test the modulatory effect of flavonoids on important transport systems at blood-brain barrier and neurons. Using *in vivo* supplementation this work intended to analyse flavonoids metabolic and cognitive effects and explore the potential of flavonoids as neuroinflammatory modulators. Also, this work intended to clarify if flavonoids were able to modulate microglia phenotype and consequent changes in synaptic plasticity.

This thesis demonstrated the impact of flavonoids on glucose uptake at blood-brain barrier (BBB). The modulatory effects of flavonoids occurred in a structural dependent way. The flavan-3-ols catechin and epicatechin did not have any effect on glucose uptake. However their methylated forms were able to increase this uptake. The effect of flavonols was in a different direction: both quercetin and miricetin decreased glucose uptake in a concentration-dependent manner. However, the glucuronic acid conjugated of quercetin, produced no effect on glucose uptake in BBB, supporting the role of metabolization on flavonoids effects. Moreover, at dopaminergic neurons, organic cations uptake, showed to be modulated by flavonoids and by its metabolites also in a structural dependent way. Flavan-3-ols inhibit [^{3}H]-MPP$^+$ uptake while naringenin, hesperitin, quercetin and metabolites increased this uptake. Flavonoids may be suggested as beneficial contributors in restoring homeostasis in dopamine disorders. Rodent

supplementation with an anthocyanin blackberry extract (BE) also showed influence of flavonoids in both dopaminergic and glucose systems.

Wistar rats' supplementation with a blackberry extract was also able to counteract some of neuroinflammatory effects induced by a high-fat diet. *In vivo* BE supplementation was also able to increase the expression of synaptic related proteins. Microglia phenotype has a role both in neuroinflammation and maintenance of neuronal synaptic homeostasis and seemed slightly affected by anthocyanins. Flavonoids interaction with communication microglia-neuron was also suggested by this work, as fractalkine expression appeared constantly altered by anthocyanins. This work presented some biological explanations which help to determine and understand the efficacy of flavonoids. Unrevealing new targets of flavonoids action may contribute to readdress future interventional studies.

RESUMO

Os flavonoides têm sido apontados como compostos promissores na prevenção do declínio cognitivo, característico do envelhecimento e de doenças neurodegenerativas. Sendo sintetizados pelo metabolismo secundário das plantas, os flavonoides estão presentes numa enorme variedade de alimentos e bebidas de origem vegetal como hortofrutícolas, chá, cacau e vinho. Estudos epidemiológicos tem associado o consumo de alimentos ricos em flavonoides com um menor risco de declínio cognitivo. Alguns dos seus mecanismos de ação já foram descritos mas muitos outros permanecem ainda por explorar. O presente trabalho pretendeu testar os efeitos dos flavonoides na modulação de importantes sistemas de transporte ao nível da barreira hemo-encefálica e em neurónios. Recorrendo a modelos de suplementação *in vivo*, este trabalho analisou os efeitos metabólicos e cognitivos dos flavonoides e explorou o seu potencial como modulador da neuroinflamação. Este trabalho pretendeu ainda clarificar se os flavonoides teriam ação sobre o fenótipo da micróglia e consequentes alterações na plasticidade sináptica.

Esta tese demonstrou o impacto dos flavonoides na captação de glicose pela barreira hemo-encefálica (BHE). A capacidade de modular este transporte mostrou ser dependente da estrutura dos flavonoides. Os flavanóis catequina e epicatequina não tiveram efeito na captação de glicose. Contudo, as suas formas metiladas aumentaram esta captação. O efeito dos flavonóis ocorreu na direção oposta: tanto a quercetina como a miricetina diminuíram a captação de glicose de uma forma dependente da concentração. No entanto, o conjugado glicuronilado da quercetina não teve qualquer efeito, sustentando o papel da metabolização no efeito metabólico dos flavonoides. Também em neurónios dopaminérgicos, os flavonoides modulam um importante sistema de transporte, o da captação de catiões orgânicos, de forma dependente

da estrutura. Compostos pertencentes à classe dos flavanóis inibem a captação de [^{3}H]-MPP$^+$ enquanto que naringenina, hesperitina, quercetina e seus metabolitos aumentam esta captação. Deste modo, os flavonoides parecem ter um papel benéfico no restabelecimento da homeostasia em situações de desequilíbrio dopaminérgico. A suplementação em ratos Wistar com extrato de amora (EA) rico em antocianinas também demonstrou a influência dos flavonoides quer no metabolismo da dopamina como da glicose.

A suplementação destes animais com um EA foi também capaz de prevenir alguns dos efeitos neuroinflamatórios induzidos pela dieta hiperlípidica e hipersacarídica. A suplementação com EA foi ainda capaz de induzir a expressão de proteínas relacionadas com a transmissão sináptica. O fenótipo da micróglia, que tem um papel importante quer na neuroinflamação quer na manutenção da homeostasia sináptica, foi ligeiramente afetado pela presença das antocianinas. A interação dos flavonoides com a comunicação entre a microglia e os neurónios foi também suportada por estes resultados em que a expressão da fractalkine aparece constantemente alterada pelas antocianinas. Este trabalho apresenta algumas explicações biológicas que ajudam a determinar e compreender a eficácia dos flavonoides. A compreensão e a revelação de novos alvos fisiológicos para a ação dos flavonoides pode redirecionar futuros estudos intervencionais.

CHAPTER I

Introduction

Aim

"Let food be your medicine and medicine be your food" was a quote from Hippocrates 460-370 B.C. who first raised the awareness of importance of diet in the maintenance and recuperation of health. The notion of diet specifically as brain function modulator is attracting interest by academics and non-academics that had recurred to herbal supplements and drugs as cognitive enhancers or as a way to delay cognitive decline (Maslen, Faulmuller et al. 2014, Muralidhara 2015). Besides pharmacological drugs, with known effects on brain function, the role of particular nutrients as coffee and glucose are also well-studied (Lieberman 2001, van der Zwaluw, van de Rest et al. 2015). Dietary fats is another subject of intense scrutiny where high-fat -obesogenic- diets have been associated with long-term changes in neurotransmission and brain plasticity, resulting in cognitive dysfunction (Kanoski and Davidson 2011, Sullivan, Nousen et al. 2014). Moreover, adherence to a healthy diet patterns as Mediterranean diet results in better cognitive outcomes and a reduced risk for dementia (Solfrizzi and Panza 2014). In support of these large population studies current bench studies are determining the pathways whereby individual elements of diet - as polyphenols, or specifically flavonoids- can influence brain function.

Flavonoids, classes and chemical structure

Flavonoids are polyphenolic compounds synthetized by secondary metabolism of plants. Their primordial roles in plants are protection, against UV radiation, pathogenic agents and predators, and attractiveness for pollination. These chemicals are not required for the immediate survival of the plant but increase its chance to survival. Their ecological roles in plant life and the molecular and biochemical similarities between plants biology and higher animals

may help to explain why these phytochemicals affect the human central nervous system (CNS) (Kennedy and Wightman 2011). Interaction with central and peripheral nervous systems can be a way of feeding dissuasion to potential herbivores, and in regard to this, these compounds evolved to act as neurotransmitters agonist or antagonists or neurotransmitter systems as well as forming structural analogous of endogenous hormones (Kennedy and Wightman 2011). However the question of why and how flavonoids affect brain function is far from being answered. Its chemical structure can open some clues.

Structurally, flavonoids are a subclass of polyphenols. The basic flavonoid structure is the flavan nucleus, which consists of a C6-C3-C6 structure constituted by two aromatic rings linked by a heterocyclic ring, labelled A, B and C (Figure 1). The various classes of flavonoids differ in the level of oxidation and pattern of substitution at the C ring, while individual compounds within a class differ in the arrangements of hydroxyl, methoxyl and glycosidic side groups (Table 1) (Heim, Tagliaferro et al. 2002, Beecher 2003).

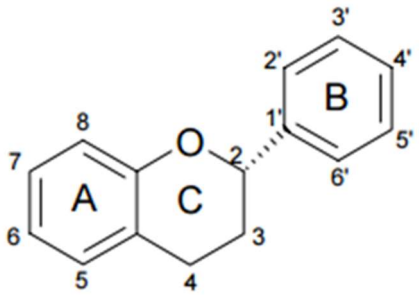


Figure 1- Flavan nucleus structure.

Until date more than 8000 flavonoids were already identified, and they can be classified into 14 classes accordingly to their chemical structure. Six of these classes have received special attention by scientific community (See Figure 2): in particular (1) flavonols (e.g., kaempferol, quercetin), which are found in onions, leeks, and broccoli; (2) flavones (e.g., apigenin, luteolin), which are found in parsley and celery; (3) isoflavones (e.g., daidzein, genistein), which are mainly

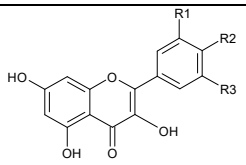
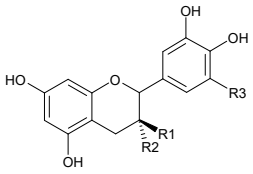
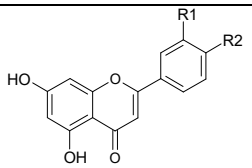
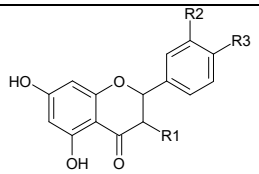
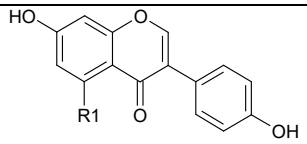
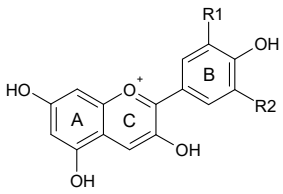
found in soy and soy products; (4) flavanones (e.g., hesperetin, naringenin), which are mainly found in citrus fruit and tomatoes; (5) flavan-3-ols (e.g., (+)-catechin, (−)-epicatechin, epigallocatechin, epigallocatechin gallate (EGCG)), which are abundant in green tea, red wine, and chocolate; and (6) anthocyanidins (e.g., pelargonidin, cyanidin, malvidin), whose sources include red wine and berry fruits(Heim, Tagliaferro et al. 2002, Egert and Rimbach 2011, Williams and Spencer 2012).

Flavonoids chemical structure make them natural free radical scavengers and from many years they were studied for their antioxidant properties (Rice-Evans, Miller et al. 1996). Contributing to this antioxidant nature are the presence of a B-ring catechol group capable of readily donating hydrogen and stabilize a radical specie (Rice-Evans, Miller et al. 1996). Other important structural features for antioxidant nature include the presence of 2,3 unsaturation in conjugation with a 4-oxo- function in the C-ring and the presence of functional groups capable of binding transition metal ions, such as iron and copper (Bors, Heller et al. 1990, Heim, Tagliaferro et al. 2002, Williams, Spencer et al. 2004) .

However, as they cannot be regenerated as other antioxidants like ascorbic acid and α-tocopherol and because their concentrations are lower when compared with these molecules, other mechanisms arise to explain their biological effects (Williams, Spencer et al. 2004). These mechanisms can involve interaction with several receptors and signaling cascades, mainly Pi3K/Akt and MAP Kinase pathways that lead to regulation of transcription factors and gene expression. This includes several genes related to enzymatic activity, as those involved in biotransformation and antioxidant activity. Flavonoids have also the ability to regulate several transport systems or substrates at different membrane levels. They can also modulate expression of genes involved in cell cycle regulation, cell proliferation, angiogenesis, inflammation and even in cognitive function. Also they are known to induce beneficial effects on cardiovascular system affecting both peripheral and cerebral blood flow (Youdim, Spencer et al.

2002, Vauzour, Vafeiadou et al. 2008, Martel, Monteiro et al. 2010, Visioli, De La Lastra et al. 2011, Williams and Spencer 2012)

Table 1- Major classes of flavonoids: examples, chemical structure, and main dietary sources. Adapted from Negrão et al, 2009 (Negrão and Faria 2009).

Class of flavonoids	Molecular Structure	Functional groups and examples	Food sources
Flavonols		R1=H; R2=OH; R3=H: kaempferol R1=OH; R2=OH; R3=H:quercetin R1=OH; R2=OH; R3=OH: myricetin	Onions, apples, curly kale, leek
Flavan-3-ols		R1=H; R2=OH; R3=H: (+)-catechin R1=OH; R2=H; R3=H: (-)-epicatechin R1=OH; R2=OH; R3=OH: (+)-gallocatechin	Chocolate, green tea, beans, cherry, grape seeds, red wine
Flavones		R1=H; R2=OH: apigenin R1=OH; R2=OH:luteolin	Parsley, celery, capsicum pepper
Flavanones		R1=H; R2=H; R3=OH: naringenin R1=OH; R2=OH; R3=OMe: hesperetin	Orange juice, grapefuit juice, lemon juice
Isoflavones		R1=H: daidzein R2=OH: genistein	Soy flour, soy beans, soymilk
Anthocyanidins		R1=H; R2=H: pelargonidin R1=OH; R2=H: cyanidin R1=OH; R2=OH: delphinidin R1=OMe; R2=OH: petunidin R1=OMe; R2=OMe: malvidin	Aubergine, blackberry, blackcurrant, blueberry

Flavonoids intake

Polyphenols are widespread, virtually present in all foods and beverages of plant origin, such as fruits, vegetables, tea, cocoa and wine. It is estimated that two thirds of total polyphenols ingested are flavonoids. However, for several reasons, it is extremely difficult to estimate the daily average intake of flavonoids, such as the extensive diversity of chemical structures that makes the estimation of flavonoid content in foods complex, the analytical method used for these estimations, variation of content with geographical region, harvesting and season, as well as dietary habits of people. Few estimations of dietary intake are available and the attained values are somehow different. Scalbert and Williamson have already proposed that the total dietary intake of polyphenols is about 1 g/day (Scalbert and Williamson 2000).

Recently, combined data from European Food Safety Authority (EFSA) and FLAVIOLA – Flavonol Food composition database which contains food composition data for approximately 3,000 food items was compiled to estimate the flavonoid intake in European countries. Mean intake of total flavonoids in Europe was found to be around 428 mg/day. Mean intake of most flavonoids in Europe is broadly comparable with intake in Australia or with observational studies conducted in the US and Europe (Vogiatzoglou, Mulligan et al. 2015). However, mean intake of anthocyanidins in some countries (Finland, Latvia and France) was similar or higher (19 mg/day) than intake in Nurses Health Study II (10.9 mg/day), or in the UK twin study (17.8 mg/day) where high anthocyanidins intake was associated with a reduced risk of myocardial infarction or vascular function respectively (Jennings, Welch et al. 2012, Cassidy, Mukamal et al. 2013).

Biokinetic of flavonoids

Absorption and distribution and biotransformation

For several years flavonoids bioavailability was considered low. With exception of flavan-3-ols subclasse, flavonoids in nature are mainly glycosides (Aherne and O'Brien 2002). Glycosides have considerable size and planar structure with low lipophilicity which turns its absorption difficult

(Walle 2004). Although intact anthocyanin glycosides have been detected in circulation, most glycated flavonoids are hydrolysed to their aglicon form by intestinal enzymes prior to absorption (Nurmi, Mursu et al. 2009). Two enzymes are involved in this deglycosylation process: lactase phloridzin hydrolase (LPH) and cytosolic β-glucosidase (CBG). The first is present in the luminal side of enterocytes and aglicones resultants from their action are able to easily cross the membrane or undergo further biotransformation by colonic microbiota. CBG is present inside the enterocyte, which implies the need of the flavonoid to cross apical cell membrane (Day, Canada et al. 2000, Nemeth, Plumb et al. 2003). Glucose transporters have been proposed as implicated in this process (Faria, Pestana et al. 2009). The need for deglicosylation, active transport into the cells or the limited absorption ability of colon limits the amount of flavonoids expected to be found in plasma.

In fact, some intervencional studies have shown great variability in flavonoids bioavailability (Williamson and Manach 2005). Several factors can influence this bioavailability: the presence of fiber, macro- and micronutrients, gastrointestinal transit time, and gut microbiota. Notably, the food matrix can also impact the degree of flavonoid bioaccessibility; also proanthocyanidins and other flavonoid polymers must be broken down into monomeric or dimeric units before they can be absorbed (Manach, Scalbert et al. 2004, Manach, Williamson et al. 2005). Flavonoids absorption has been discussed as occurring in further places other than at intestinal level. Anthocyanins have been shown to be absorbed at gastric level and absorption at oral cavity has also been proposed (Faria, Fernandes et al. 2014). Furthermore, flavonoids can undergo further metabolization by colon microbiota and this colonic metabolites may also be absorbed and consequently be implicated in biological activity (Faria, Fernandes et al. 2014).

Flavonoids undergo extensive conjugation by Phase II enzymes producing methoxylated, glucuronidated, and sulphated compounds during and after absorption process. The plasma concentration of unconjugated flavonoid is usually very low, implying an extensive conjugation

of the absorbed flavonoid. The extent of this biotransformation suggests that either metabolites, the parent glucoside or free form of the compound can be responsible for flavonoid bioactivity (Corcoran, McKay et al. 2012). This issue will be taken in account in the next chapters.

Flavonoids access to the brain

To exert activity, flavonoids do not necessarily need to reach the brain. They can have biological function even without being absorbed at intestinal level, by changing gut microbiota or inducing local stimulus that may act at brain level (Foster and McVey Neufeld 2013, Faria, Fernandes et al. 2014). Notwithstanding, flavonoids can have direct action on nervous system and this will, in part, depend on the extension of their accessibility to the brain. As presented above flavonoids are indeed bioavailable at intestinal level and reach the plasmatic circulation where they can find another barrier before reach the brain. This blood-brain barrier (BBB) is a restraining barrier of brain capillary endothelial cells tightly connected, limiting the access to most small polar molecules and macromolecules to the brain (Abbott, Patabendige et al. 2010). Besides fulfilling a protective role by severely limiting the movement of substances into the brain, BBB has also the crucial role of supplying essential nutrients, hormones, drugs or xenobiotics to the brain (Palmer 2010). Specific transport systems therefore are expressed in the BBB to ensure an adequate supply of these substances. Literature about flavonoids ability to interact with nutrient supply at this level was scarce or inexistent.

Besides the potential to interact locally at BBB, permeability of BBB to flavonoids has been already demonstrated in several models. Most of the flavonoids and respective metabolites tested are able to cross the different models of BBB (Youdim, Dobbie et al. 2003, Faria, Pestana et al. 2010, Faria, Meireles et al. 2014, Yang, Bai et al. 2014). In agreement, several animal studies have detected some flavonoids and metabolites on brain tissue. For example, epicatechin glucuronide and 3'-O-methyl epicatechin glucuronide have been found in the brain of rats after oral supplementation with epicatechin for 1, 5 or 10 days (Abd El Mohsen, Kuhnle et

al. 2002). Similar observations have been made for the flavonones: naringenin and hesperetin, which have been detected in brain tissue after intravenous administration (Tsai and Chen 2000, Lin, Hou et al. 2014) and for EGCG (Suganuma, Okabe et al. 1998) and anthocyanins (Talavéra, Felgines et al. 2005, El Mohsen, Marks et al. 2006) after oral ingestion. Recently, a dose-dependent accumulation of several flavonoids was seen in cerebellum and frontal cortex in weanling pigs (Chen, Kritchevsky et al. 2015). Curiously there is also evidence of flavonoids brain accumulation after long-term exposure (Ferruzzi, Lobo et al. 2009), which may contribute to increase its bioactive potential.

Evidence from protective role of flavonoids

The association of fruits and vegetables consumption with lower risk of development of cardiovascular diseases has been strongly supported by epidemiological data as reviewed by Wang *et al* (Wang, Ouyang et al. 2014). Also, several studies have reported an association of increased intake of vegetables with a lower risk of dementia and slower rates of cognitive decline in older age (Loef and Walach 2012). Flavonoids may be in part responsible for some of these protector effects (Hooper, Kroon et al. 2008). Indeed some epidemiological studies have suggested that flavonoids play a protective role against cognitive decline and Alzheimer disease (Engelhart, Geerlings et al. 2002). The PAQUID Study was one of the first epidemiological studies to demonstrate such associations (Commenges, Scotet et al. 2000, Letenneur, Proust-Lima et al. 2007, Schaffer, Asseburg et al. 2012). Recently Kesse-Guyot and colleagues showed an association between polyphenols intake and better performance in language and verbal memory tasks (Kesse-Guyot, Fezeu et al. 2012). Also a positive association between flavonoids intake and prevention of Parkinson's disease has been found (Gao, Cassidy et al. 2012) expanding the spectrum of favorable effects of their consumption.

Furthermore, some evidences from interventional studies are also available: elderly adults consuming blueberry juice showed some neurocognitive benefits through memory improvement

(Krikorian, Shidler et al. 2010) and supplementation with concord juice enhanced cognitive function in elderly adults with early memory decline (Krikorian, Nash et al. 2010). Also global cognitive function was significantly better after 8-wk consumption of flavanone-rich juice than after 8-wk consumption of the low-flavanone control (Kean, Lamport et al. 2015).

Epidemiological studies gave rise to many investigations using animal models. Some studies have demonstrated, for instance, that supplementation with berry extracts with high levels of anthocyanins or other polyphenols can reverse brain insult and age-related cognitive decrements in rodents (Willis, Shukitt-Hale et al. 2009). Supplementation with these extracts resulted in improved memory, inducing better performance at Morris water maze and object recognition (Joseph, Shukitt-Hale et al. 2009, Shukitt-Hale, Cheng et al. 2009, Rendeiro, Vauzour et al. 2012).

Flavonoids have also been presented as neuroinflammation modulators (Spencer, Vafeiadou et al. 2012). Neuroinflammation was classically seen as a result of microglia activation cause or consequence of neuronal death, which is a common final event in neurodegenerative diseases (Rodrigues, Sanberg et al. 2014). This neuroinflammation contributes to the neurodegenerative cascade leading to neurodegenerative pathologies as Alzheimer disease, Parkinson's disease and clinical dementia (Hirsch and Hunot 2009, Gorelick 2010). Flavonoid interventions that aimed to target this feature have received particular interest and promising results as reviewed by Jang et al (Jang and Johnson 2010) .

Previous literature show that flavonoids action involves in part decreases in oxidative/inflammatory stress signaling and also that hormetic effects may be involved in protective mechanisms of neurons against oxidative and inflammatory stressors. Nevertheless, many of the mechanisms underpinning their beneficial effects remain to be elucidated (Spencer 2009, Vauzour 2012), and this thesis intended to provide further insights into this field.

This study aimed to explore the potential of specific food components, the flavonoids, on brain health, hypothesising that they would represent a positive approach to maintain brain homeostasis and function.

In order to accomplish that, several specific goals were defined:

- Test the effect of flavonoids on modulation of glucose uptake at blood-brain barrier level;
- Determine whether flavonoids were able to modulate important transport systems in neurons;
- Analyse flavonoids metabolic and cognitive effects after *in vivo* supplementation, in a context of standard and high-fat diet;
- Explore the potential of flavonoids as neuroinflammatory modulators;
- Determine whether flavonoids were able to change microglia phenotype and consequently change synaptic plasticity.

CHAPTER II

“ Characterization and modulation of glucose uptake in a human blood–brain barrier model”

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ORIGINAL ARTICLES

Characterization and Modulation of Glucose Uptake in a Human Blood–Brain Barrier Model

Manuela Meireles · Fátima Martel · João Araújo · Celestino Santos-Buelga · Susana Gonzalez-Manzano · Montserrat Dueñas · Victor de Freitas · Nuno Mateus · Conceição Calhau · Ana Faria

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Abstract The blood–brain barrier (BBB) plays a key role in limiting and regulating glucose access to glial and neuronal cells. In this work glucose uptake on a human BBB cell model (the hCMEC/D3 cell line) was characterized. The influence of some hormones and diet components on glucose uptake was also studied. ^{3}H-2-deoxy-D-glucose ([^{3}H]-DG) uptake for hCMEC/D3 cells was evaluated in the presence or absence of tested compounds. [^{3}H]-DG uptake was sodium- and energy-independent. [^{3}H]-DG uptake was regulated by Ca^{2+} and calmodulin but not by MAPK kinase pathways. PKC, PKA and protein tyrosine kinase also seem to be involved in glucose uptake modulation. Progesterone and estrone were found to decrease ^{3}H-DG uptake. Catechin and epicatechin did not have any effect, but their methylated metabolites increased [^{3}H]-DG uptake. Quercetin and myricetin decreased [^{3}H]-DG uptake, and glucuronic acid-conjugated quercetin did not have any effect. These cells expressed GLUT1, GLUT3 and SGLT1 mRNA.

Keywords Blood–brain barrier · Flavonoid · Glucose · Hormone · Signaling pathway

M. Meireles · F. Martel · J. Araújo · C. Calhau · A. Faria (✉)
Department of Biochemistry (U38.FCT), Faculty of Medicine, University of Porto, Al. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal
e-mail: anafaria@med.up.pt

C. Santos-Buelga · S. Gonzalez-Manzano · M. Dueñas · A. Faria
Grupo de Investigación en Polifenoles (GIP-USAL), Universidad de Salamanca, 37007 Salamanca, Spain

V. de Freitas · N. Mateus
Chemistry Investigation Center (CIQ), Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal

C. Calhau
CINTESIS—Center for Research in Health Technologies and Information Systems, University of Porto, 4200-319 Porto, Portugal

A. Faria
Faculty of Nutrition and Food Sciences, University of Porto, 4200-465 Porto, Portugal

Introduction

The brain requires a constant and substantial energy supply to maintain its main functions. Beyond infancy, and under normal conditions, brain energy requirements are met almost exclusively by the breakdown of glucose. During hypoglycemia, other tissues will stop using glucose altogether in order to increase glucose availability to the brain (Owen and Sunram-Lea 2011).

Compared to other organs, the brain is particularly vulnerable to small and transient changes in its energy supply. Interrupted delivery leads within seconds to unconsciousness and within minutes may cause irreparable brain damage, which makes brain function highly dependent on the availability and metabolism of glucose and oxygen resources (Owen and Sunram-Lea 2011).

Free exchange between the blood and interstitial fluid occurs in nearly all organs of the body with the exception of the capillaries in the brain, which have evolved to constrain the movement of molecules and cells between the blood and brain. This characteristic, besides fulfilling a protective role in providing a natural defense against toxic or infective agents circulating in the blood, has also the crucial role of supplying essential nutrients, hormones and drugs to the brain and eliminating metabolites from the brain (Abbott et al. 2010). The blood–brain barrier (BBB)

is made up of three cell types, endothelial cells, astrocytes and pericytes; and the barrier properties are conferred by cell adhesion molecules that allow endothelial cells to form tight junctions.

Brain glucose levels are maintained through the balance of glucose's utilization in neural cells and its entry from the peripheral circulation via enhanced cerebral blood flow (Kiyatkin and Lenoir 2012). Since most of the energy required to sustain normal brain function is provided by blood glucose, glucose utilization in the brain may be limited by BBB glucose transport (Maher et al. 1994).

Glucose transport through membranes must involve a transport system, and thus, it is subject to its expression, function and regulation with consequences to brain glucose availability. Glucose transporter type 1 deficiency syndrome is a treatable epileptic encephalopathy, caused by a defect of glucose transport mediated by GLUT1 across the BBB and into brain cells. It is characterized by an infantile-onset epileptic encephalopathy associated with delayed neurological development, deceleration of head growth, acquired microcephaly, incoordination and spasticity (Verrotti et al. 2012).

Several substances, e.g., dietary components, hormones, therapeutic drugs and drugs of abuse, have been shown to influence different transporters at different levels (e.g., enterocyte, colon, placenta) (Araujo et al. 2010; Goncalves et al. 2009, 2011; Keating et al. 2007, 2009; Martel et al. 2010). Particularly, glucose transport was shown to be modified by flavonoids, vitamins and drugs of abuse (Araujo et al. 2008; Faria et al. 2009; Keating et al. 2009; Martel et al. 2010).

Flavonoids (a major group of polyphenols) are ubiquitously found in nature and important components in the human diet. Epidemiological and dietary intervention studies in humans and other animals have indicated that flavonoid consumption may be capable of promoting neuronal health (Aquilano et al. 2008; Lau et al. 2006; Singh et al. 2008; Spencer 2009b, c; Spencer et al. 2009). However, flavonoids' mechanisms of action are not fully understood: whether they have a direct effect or interfere with cellular mechanisms causing biological effects is not completely clarified.

Because the BBB is a critical barrier in controlling brain glucose homeostasis and because glucose transport is influenced by several substances, increasing our knowledge of glucose uptake characterization in a human BBB model and its modulation by hormonal and dietary compounds is urgent.

The development of an immortalized cell line of human brain capillary endothelial cells which display many of the properties of the BBB cells in vivo provides a good model for glucose transport across the BBB in vitro (Ohtsuki et al. 2013; Weksler et al. 2005).

Materials and Methods

Chemicals

^{3}H-2-deoxy-D-glucose ([^{3}H]-DG, specific activity 40–50 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO); (+)-catechin, (−)-epicatechin, quercetin, myricetin, neomycin, 4′,5,7-trihydroxyisoflavone (genistein), 5,5′-dimethyl-BAPTA-AM, calmidazolium, chelerythrine chloride, H-89 dihydrochloride hydrate, KN-62, dinitrophenol, phloridzin, rapamycin, LY294002, progesterone, testosterone, estradiol, estrone, corticosterone, pregnanediol, aldosterone, penicillin G, amphotericin B, streptomycin, HEPES, trypsin–EDTA and collagen type I from rat tail were from Sigma-Aldrich (Madrid, Spain); PD 98058, SB 203580 and SP600125 were from Research Biochemicals International (Natick, MA); dimethylsulfoxide (DMSO) and triton X-100 were from Merck (Darmstadt, Germany); fetal bovine serum (FBS), basic fibroblast growth factor (FGF) and Hanks balanced salt solution (HBSS) were from GIBCO (Barcelona, Spain); EBM-2 medium, vascular endothelial growth factor (VEGF), IGF-I, epidermal growth factor (EGF), basic FGF, hydrocortisone, ascorbate and gentamycin were from Clonetics (Wokingham, UK).

3′-*O*-Methylcatechin and 3′- and 4′-*O*-methylepicatechin were prepared by hemisynthesis as described (Gonzalez-Manzano et al. 2009). Quercetin-3-*O*-glucuronide was isolated from green bean pods as reported (Duenas et al. 2011).

Cell and Culture Conditions

The hCMEC/D3 cell line was kindly supplied by Dr. Pierre-Olivier Couraud (INSERM U.567, Université René Descartes, Paris, France). Cells were maintained in a humidified atmosphere of 5 % CO_2–95 % air at 37 °C, between passages 26 and 34. Cells were grown in EBM-2 medium supplemented with VEGF, IGF-I, EGF, basic FGF, hydrocortisone, ascorbate, gentamycin and 2.5 % FBS as recommended by the manufacturer, as well as 100 U/ml penicillin G, 0.25 mg/ml amphotericin B and 100 mg/ml streptomycin. The cell medium was changed every 48 h, and cells reached confluence after 5–6 days in culture. For subculturing, cells were dissociated with 0.25 % trypsin–EDTA, diluted 1:5 and subcultured in Petri dishes collagen-coated with a 21-cm^{2} growth area (Corning Costar, Badhoevedorp, the Netherlands).

For the experiments, cells were seeded on 24-well collagen-coated plates (Corning Costar). All experiments were performed 7–9 days after initial seeding.

[^{3}H]DG Uptake

The culture medium was aspirated, and cells were washed with 0.3 ml buffer at 37 °C. Then, hCMEC/D3 cells were deprived of glucose for 2 h (preincubation period) by incubation with glucose-free HEPES buffered saline (containing [in mM] 140 NaCl, 5 KCl, 1 pyruvate, 2.5 $MgSO_4$, 1 $CaCl_2$ and 20 HEPES, pH 7.4). Uptake was initiated by the addition of 0.3 ml medium at 37 °C containing 50 nM [^{3}H]-DG. At the end of the incubation period, incubation was stopped by placing the cells on ice and rinsing them with 0.5 ml ice-cold buffer. Cells were then solubilized with 0.3 ml 0.1 % (v/v) Triton X-100 (in 5 mM Tris–HCl, pH 7.4) and placed at 37 °C overnight. Radioactivity in the cells was measured by liquid scintillation counting. Compounds to be tested were present during the last 50 or 30 min of the preincubation period and during the incubation period. Controls were made with 0.1 % of solvent.

Quantitative RT-PCR

RNA was extracted from the hCMEC/D3 cells using the Tripure Isolation Reagent (Roche, Indianapolis, IN), according to the manufacturer's instructions. RNA was dissolved in water (diethylpyrocarbonate-treated) and stored at −80 °C.

Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA), and 0.5 µg of the resulting DNA-free RNA was reverse-transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen) in 20 µl of final reaction volume, according to the manufacturer's instructions. The resulting cDNA was treated with RNase H (Invitrogen) to degrade unreacted RNA. For real-time quantitative PCR, 2 µl of the 20 µl reverse transcription reaction mixture was used. For the calibration curve, hCMEC/D3 standard cDNA was diluted in six different concentrations.

QueryReal-time PCR was carried out using a LightCycler (Roche, Nutley, NJ). Twenty-microliter reactions were set up in microcapillary tubes using 0.5 µM of each primer and 4 µl of SYBRGreen master mix (LightCycler FastStart DNA MasterPlus SYBR Green I; Roche, Indianapolis, IN). Cycling conditions were as follows: denaturation (95 °C for 5 min), amplification and quantification (95 °C for 10 s, annealing temperature [AT] for 5 s and 72 °C for 10 s, with a single fluorescence measurement at the end of the 72 °C for a 10-s segment) repeated 45 times, a melting curve program ([AT+10] °C for 15 s and 95 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement) and a cooling step to 40 °C. Annealing temperatures and sequences of primers are indicated in Table 1. Data were analyzed using LightCycler software. Results are presented as the expression of the gene of interest relative to hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Protein Determination

The protein content of cell monolayers was determined by Bradford's (1976) method, using human serum albumin as standard.

Statistical Analysis

All assays were performed at least in two independent experiments with $n \geq 3$ replicates. Values are expressed as the arithmetic mean ± SEM. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance, followed by the Bonferroni test. For comparison between two groups, Student's t test was used. Differences were considered significant when $p < 0.05$.

Results

Characterization of [^{3}H]-DG Uptake by hCMEC/D3 Cells

Cellular uptake of [^{3}H]-DG was determined using hCMEC/D3 cells as a model of the human BBB. [^{3}H]-DG was taken up by the cells in a time-dependent way, and uptake was linear with time for up to 5 min of incubation (data not shown). Thus, all subsequent experiments were performed using a 1-min incubation period, in the presence or absence of compounds, in order to determine initial rates of uptake.

Sodium and Energy Dependence of [^{3}H]-DG Uptake

Replacement of NaCl by LiCl in both the preincubation and incubation media significantly increased [^{3}H]-DG

Table 1 Primer sequences and annealing temperatures used in qRT-PCR

Gene name	Primer sequence (5′–3′)	AT (°C)
GLUT1	GAT GAT GCG GGA GAA GAA GGT	65
	ACA GCG TTG ATG CCA GAC AG	
GLUT2	CAG GAC TAT ATT GTG GGC TAA	65
	CTG ATG AAA AGT GCC AAG T	
GLUT3	CTT CCC CTC CGC TGC TCA CTA	64
	CAA AAG TCC TGC CAC GGG TCT	
SGLT1	TGG CAA TCA CTG CCC TTT A	60
	TGC AAG GTG TCC GTG TAA AT	
HPRT	GGT CAA GGT CGC AAG C	65
	GGG CAT ATC CTA CAA CAA ACT	

uptake. However, when NaCl was replaced by choline chloride (CoCl), no significant changes were induced in [^{3}H]-DG uptake (Fig. 1a).

Dinitrophenol, which inhibits ATP production, and phloridzin, an inhibitor of the sodium-dependent glucose transporter, were also tested in order to characterize [^{3}H]-DG uptake in hCMEC/D3 cells (Fig. 1b). No differences in [^{3}H]-DG uptake were found with either of these compounds, revealing that [^{3}H]-DG is energy-independent and that the involvement of SGLT1 in this transport can be excluded.

mRNA Expression of Glucose Transporters

The mRNA expression of glucose transporters in hCMEC/D3 cells was analyzed by qRT-PCR. As previously described (Cardoso et al. 2011; Uchida et al. 2011), GLUT1 was the most expressed glucose transporter; but interestingly, SGLT1 and GLUT3 were also present in this cell line. GLUT2 was not expressed (Fig. 2).

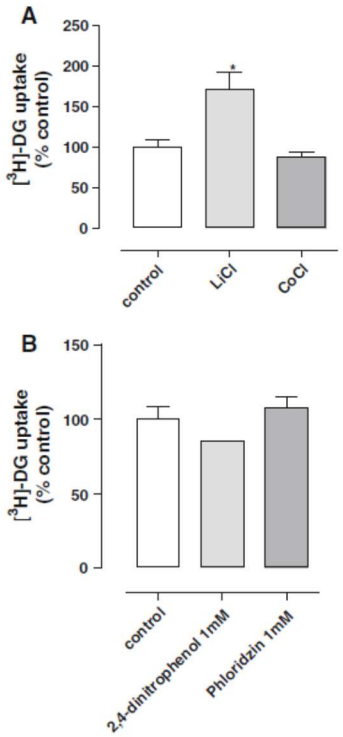

Fig. 1 **a** Effect of Na^{+} substitution by LiCl and choline chloride (CoCl) on [^{3}H]-DG uptake by hCMEC/D3 cells. Cells were preincubated for 2 h with glucose-free buffer and then incubated with [^{3}H]-DG (50 nM) for 1 min. The tested compounds were present in the last 30 min of preincubation and in the incubation. **b** Effect of dinitrophenol and phloridzin on [^{3}H]-DG uptake by hCMEC/D3 cells. Cells were preincubated for 2 h with glucose-free buffer and then incubated with [^{3}H]-DG (50 nM) for 1 min. The tested compounds were present in the last 30 min of preincubation and in the incubation. $^{*}p < 0.05$ versus control

Regulation of [^{3}H]-DG Uptake by Intracellular Signaling Pathways

The intracellular signaling mechanisms regulating [^{3}H]-DG uptake in hCMEC/D3 cells were next studied. For this, the effect of a 50-min exposure of hCMEC/D3 cells to modulators of intracellular signaling pathways was assessed.

First, the role of intracellular Ca^{2+} was studied by testing the effect of the Ca^{2+} chelator BAPTA-AM and of calmidazolium, a Ca^{2+}/calmodulin (CaM) inhibitor. There was a significant decrease in glucose uptake in the presence of these compounds (Fig. 3a). These results suggested the involvement of Ca^{2+} in modulating glucose uptake. On the other hand, KN-62, a CaM-dependent protein kinase II (CaMK II) inhibitor, did not have any effect on glucose uptake in the hCMEC/D3 cell line (Fig. 3a).

The involvement of MAP kinase (MAPK) was investigated by testing the effect of specific inhibitors of MAPK, ERK1/2 (PD 98059), p38 MAPK (SB 203580) and JNK (SP 600125). None of these compounds significantly interfered with [^{3}H]-DG uptake, suggesting it is not dependent on MAPK regulation (Fig. 3b).

The involvement of protein kinase pathways was tested using chelerythrine, a selective inhibitor of PKC, and H89, a selective inhibitor of PKA. Both compounds interfered with glucose uptake but in opposite directions: inhibition of PKC led to a decrease in [^{3}H]-DG uptake and inhibition of PKA resulted in an increase in [^{3}H]-DG uptake (Fig. 3c). Genistein, an inhibitor of protein tyrosine kinases (PTKs), was also tested and found to increase [^{3}H]-DG uptake. LY294002, a known inhibitor of phosphoinositide

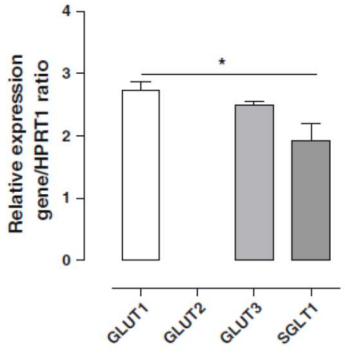

Fig. 2 Quantification of mRNA levels of GLUT1, -2, -3 and SGLT1 by qRT-PCR in hCMEC/D3 cells. Results are shown as the expression of the gene of interest relative to HPRT (arithmetic mean ± SEM). $^{*}p < 0.05$

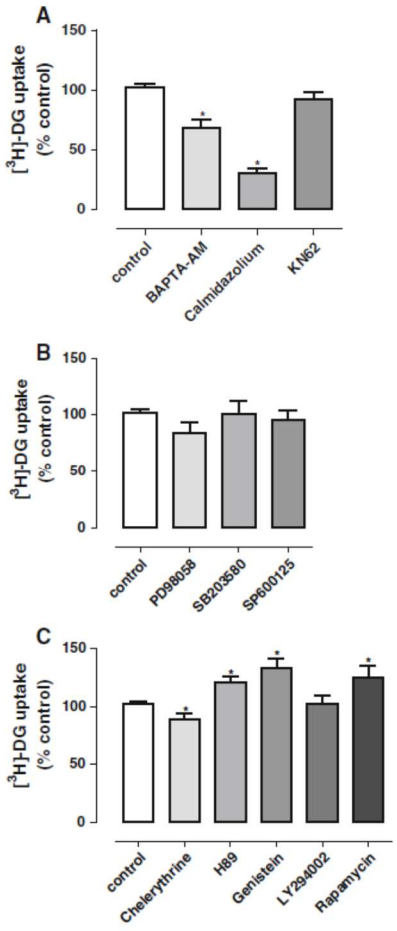

Fig. 3 Effect of several signaling pathway inhibitors on [^{3}H]-DG uptake by hCMEC/D3 cells. Cells were preincubated for 2 h with glucose-free buffer and then incubated at 37 °C with [^{3}H]-DG (50 nM) for 1 min. The tested compounds were present in the last 50 min of preincubation and in the incubation. **a** Effect of BAPTA-AM (50 μM), calmidazolium (25 μM) and KN-62 (10 μM). **b** Effect of PD 98059, SB 203580 and SP 600125 (25 μM). **c** Effect of chelerythrine (40 μM), H89 (10 μM), genistein (10 μM), LY294002 (10 μM) and rapamycin (500 nM). *$p < 0.05$ versus control

3-kinases (PI3 K), did not have any effect on [^{3}H]-DG uptake; but rapamycin, an inhibitor of the mTOR pathway, increased [^{3}H]-DG uptake in this cell line (Fig. 3c).

Hormonal Modulation of [^{3}H]-DG Uptake

In order to assess a possible endocrine modulation of ^{3}H-DG uptake, several hormones were tested: progesterone, testosterone, estradiol, estrone, corticosterone, pregnanediol and aldosterone (Table 2). Of these, only progesterone and estrone were able to influence [^{3}H]-DG uptake, decreasing it.

Nutritional Modulation of [^{3}H]-DG Uptake

A possible nutritional modulation of [^{3}H]-DG uptake was also assessed. Polyphenols are important dietary components since several beneficial neuroprotective effects have been attributed to their consumption. Catechin and epicatechin, two flavan-3-ols, as well as their methylated metabolites were tested. The unconjugated polyphenols had no effect on [^{3}H]-DG uptake, but interestingly, their methylated forms were able to increase [^{3}H]-DG uptake (Fig. 4).

Quercetin and myricetin, two flavonols, were also tested along with a quercetin–glucuronic acid conjugate (Fig. 5). The flavonols decreased [^{3}H]-DG uptake in a concentration-dependent manner, but the conjugate had no effect on [^{3}H]-DG uptake.

Table 2 Effect of several hormones on [^{3}H]-DG uptake by hCMEC/D3 cells

Compound	[^{3}H]-DG (% control)
Control	100.0 ± 2.2
Progesterone (μM)	
50	53.3 ± 12.0*
100	71.9 ± 8.59*
200	54.5 ± 15.8*
Testosterone (μM)	
50	92.4 ± 7.4
100	99.7 ± 6.7
200	112.8 ± 17.0
Estradiol (μM)	
100	82.4 ± 4.3
250	94.9 ± 11.6
500	118.7 ± 19.0
Estrone (μM)	
50	86.2 ± 3.89
100	78.8 ± 7.9
200	70.8 ± 1.6*
Corticosterone (μM)	
100	99.2 ± 7.3
Pregnanediol (μM)	
100	89.3 ± 15.3
Aldosterone (μM)	
100	102.7 ± 7.4

Cells were preincubated for 2 h with glucose-free buffer and then incubated at 37 °C with [^{3}H]-DG (50 nM) for 1 min. The tested compounds were present in the last 30 min of the preincubation period and in the incubation

* $p < 0.05$ versus control

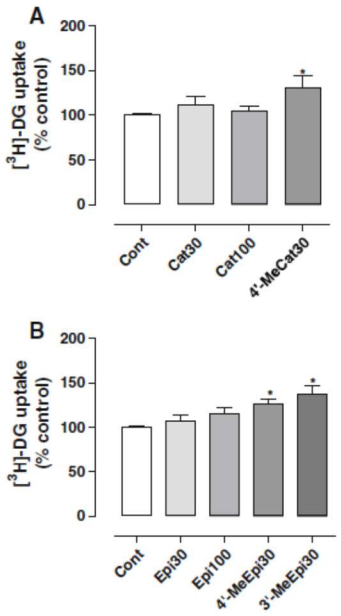

Fig. 4 Effect of catechin and epicatechin and their methylated metabolites on [^{3}H]-DG uptake by hCMEC/D3 cells. Cells were preincubated for 2 h with glucose-free buffer and then incubated at 37 °C with [^{3}H]-DG (50 nM) for 1 min. The tested compounds were present in the last 30 min of preincubation and in the incubation. **a** Effect of catechin (*Cat*, 30 and 100 μM) and of 4′-methylcatechin (*4′-MeCat*, 30 μM) on [^{3}H]-DG uptake by hCMEC/D3 cells. **b** Effect of epicatechin (*Epi*, 30 and 100 μM), 4′-methylepicatechin (*4′-MeEpi*, 30 μM) and 3′-methylepicatechin (*3′-MeEpi*, 30 μM) on [^{3}H]-DG uptake by hCMEC/D3 cells. $*p < 0.05$ versus control

Discussion

Glucose is an essential substrate to the organism, in particular to the brain as it is the main energetic substrate to neurons; therefore, glucose supply is crucial to maintain the normal functions of the brain. However, due to its hydrophilic nature, it cannot freely cross cellular membranes and, thus, needs specialized transporters to facilitate its transport. The first limiting barrier to glucose access into the brain is the BBB. Therefore, uptake of glucose at the BBB level and its modulation are of great importance since they will have consequences upon brain glucose availability.

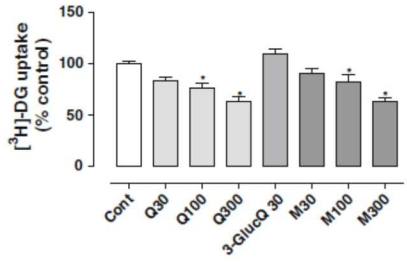

Fig. 5 Effect of quercetin (*Q*; 30, 100 and 300 μM), 3-glucuronilquercetin (*3-GlucQ*, 30 μM) and myricetin (*M*; 30, 100 and 300 μM) on [^{3}H]-DG uptake by hCMEC/D3 cells. Cells were preincubated for 2 h with glucose-free buffer and then incubated at 37 °C with [^{3}H]-DG (50 nM) for 1 min. The tested compounds were present in the last 30 min of preincubation and in the incubation. $*p < 0.05$ versus control

In this work, a human BBB cell line, hCMEC/D3, was used as a model of the BBB to study glucose uptake. This cell line is well characterized and retains most of the morphological and functional features of brain endothelial cells, even without coculture with glia cells (Ohtsuki et al. 2013; Weksler et al. 2005).

First, a functional characterization of glucose uptake into these cells was made, allowing us to conclude that glucose uptake into hCMEC/D3 cells was sodium-independent. Isosmolar substitution of extracellular NaCl by LiCl led to an increase in this uptake, but this is not consistent with Na^+ dependence and does not seem enough to support the conclusion that this transport is sodium-dependent. Importantly, taking into account the therapeutic use of lithium as a mood enhancer (Malhi et al. 2012) and the first-choice therapy for bipolar disorder, for example, we have to consider this effect on glucose uptake as potentially having a clinical expression.

In addition, the lack of effect of dinitrophenol, an inhibitor of ATP production, supports the conclusion of the energy-independent nature of glucose uptake into these cells.

According to the literature, GLUT1 is the main transporter responsible for glucose transport at the BBB level (Cardoso et al. 2011; Simpson et al. 2007; Uchida et al. 2011). However, the presence of other glucose transporters cannot be excluded. In the present work, the mRNA expression of some glucose transporters, namely, GLUT1, GLUT2, GLUT3 and the sodium-dependent glucose transporter 1 (SGLT1), was quantified; and with the exception of GLUT2, all were found to be expressed by hCMEC/D3 cells. GLUT1 had the highest expression, but surprisingly, GLUT3 also had a very high expression, not even statistically different from GLUT1. GLUT3 is usually associated with glucose uptake at the neuronal level but not at the BBB level (Cardoso et al. 2011; Simpson et al. 2007). However, a recent study described the expression of GLUT3 and GLUT14 in human brain microvessels (Uchida et al. 2011), which is in line with the finding, some years ago, of GLUT3 presence in dog BBB (Gerhart et al. 1995). Nevertheless, one could not exclude a relation of this result with the high energy requirements of these cells due to their potent proliferative nature. Moreover, in a murine cell line, the presence of GLUT8 and GLUT9 was also detected (Cura and Carruthers 2010). Because GLUT3

mRNA is present in a significant amount in hCMEC/D3 cells, it should not be excluded or not taken into consideration when studying glucose transport at the BBB. Interesting, SGLT1 also had remarkable levels of expression. This could be valuable information to fully understand glucose uptake at the BBB level, especially in situations associated with low glucose availability, where SGLT1, as a high-affinity glucose transporter (Ferraris 2001), could have an important role in determining glucose availability to the brain. In this study, DG was used as a substrate, and because this compound is not a good substrate for SGLT1, the contribution of SGLT1 for the studied transport could be neglected. This was confirmed by the functional characterization of [^{3}H]-DG uptake (see above).

Inhibitors of several pathways were studied in order to understand which intracellular signaling pathways may be involved in the regulation of glucose uptake by hCMEC/D3 cells. Recently, it was described that AMP kinase activation directs the trafficking of GLUT1 to the plasma membrane, having a direct role in regulating the glucose transport capacity of the BBB (Cura and Carruthers 2012). Also, according to the literature, Ca^{2+} appears to be involved in GLUT1 translocation and calmodulin is involved in GLUT1 stimulation (Maraldi et al. 2006).

These findings are in line with the literature as Ca^{2+} quelation and the inhibition of calmodulin interfered with [^{3}H]-DG uptake (Maraldi et al. 2006). Specific inhibitors of MAPK pathways were also used, but no effect on [^{3}H]-DG uptake was observed, excluding them from having a role in GLUT regulation. On the other hand, several protein kinases seem to be involved in this transport regulation: PKA and PTK are glucose-uptake inhibitors, and PKC is an activator of this transport. Interestingly, it is known that intracellular Ca^{2+} (Ikeda et al. 1997) and total PKC activation (Fleegal et al. 2005) increase gradually during hypoxia in BBB cells, which could positively influence glucose uptake in these cells in this extreme condition. mTOR has been negatively related with GLUT4 trafficking (Jiang et al. 2008), and in this work the mTOR inhibitor rapamycin increased glucose uptake. This could suggest a possible involvement of this factor in membrane localization of the glucose transporter.

Taking into account that hormone levels have an impact on cognition, learning and memory (Bimonte-Nelson et al. 2010; Foy et al. 2010; Maggio et al. 2012), we recognized the relevance of investigating the effect of several endogenous hormones on [^{3}H]-DG uptake by hCMEC/D3 cells. Of all of the hormones tested, only progesterone and estrone were able to influence [^{3}H]-DG uptake, by decreasing it. Progesterone has been characterized as an activator of GLUT expression in chronic treatment (Frolova et al. 2009; Medina et al. 2004), but herein the opposite effect was observed with acute treatment; one could hypothesize that this cell response could be part of a compensation mechanism. Interestingly, the two hormones that have an effect on [^{3}H]-DG uptake are present in high levels during pregnancy (Brett and Baxendale 2001), which could suggest a relationship between decreased brain glucose levels and memory impairment during this phase (Henry and Rendell 2007).

Polyphenols have received attention from the scientific community due to their beneficial health properties, in particular at the neuronal level (Gutierrez-Merino et al. 2011). Several epidemiological and dietary intervention studies in humans and other animals indicate that polyphenol consumption is important for neuronal health (Assuncao et al. 2011; Krikorian et al. 2010; Spencer 2009a; Youdim et al. 2004). Interference with glucose uptake at the neuronal level, allowing a greater influx of glucose into the brain, could be an important mechanism contributing to the beneficial effect of polyphenols for neuronal health. Nevertheless, there is evidence supporting the extensive biotransformation of polyphenols to different conjugated derivatives (*O*-methylated, glucuronides, sulfates), which would be the main circulating forms, able to reach metabolic targets (Crozier et al. 2009). Two classes of the most abundant flavonoids were tested, and different effects were achieved. The flavan-3-ols catechin and epicatechin did not have any effect on [^{3}H]-DG uptake. However, it was noted that the methylated forms of catechin and epicatechin were able to increase [^{3}H]-DG uptake. Bearing in mind that these are probably among the most abundant forms circulating in vivo, an increase of glucose uptake through the BBB should be expected. The effects of flavonols were in a different direction: both quercetin and myricetin decreased [^{3}H]-DG uptake in a concentration-dependent manner. However, the glucuronic acid conjugate of quercetin, one of the most abundant circulating metabolites of quercetin after ingestion of a quercetin-rich meal (Mullen et al. 2004), produced no effect on [^{3}H]-DG uptake by hCMEC/D3 cells, which could support a reduced impact of quercetin ingestion on glucose uptake.

Overall, this work showed that DG uptake in a human BBB model (1) is sodium- and energy-independent; (2) probably involves GLUT1 and/or GLUT3; (3) is regulated by Ca^{2+}-dependent pathways; (4) is regulated by kinase proteins such as PKC, PKA and PTK; and (5) is regulated by flavonoids but especially by their in vivo methylated metabolites.

This work is expected to contribute to the clarification of glucose transporter modulation by dietary compounds or hormones, which could be critical to supporting nutritional recommendations in the future and to understanding metabolic dysfunctions.

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References

Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ (2010) Structure and function of the blood–brain barrier. Neurobiol Dis 37:13–25

Aquilano K, Baldelli S, Rotilio G, Ciriolo MR (2008) Role of nitric oxide synthases in Parkinson's disease: a review on the antioxidant and anti-inflammatory activity of polyphenols. Neurochem Res 33:2416–2426

Araujo JR, Goncalves P, Martel F (2008) Modulation of glucose uptake in a human choriocarcinoma cell line (BeWo) by dietary bioactive compounds and drugs of abuse. J Biochem 144:177–186

Araujo JR, Goncalves P, Martel F (2010) Characterization of uptake of folates by rat and human blood–brain barrier endothelial cells. BioFactors 36:201–209

Assuncao M, Santos-Marques MJ, Carvalho F, Lukoyanov NV, Andrade JP (2011) Chronic green tea consumption prevents age-related changes in rat hippocampal formation. Neurobiol Aging 32:707–717

Bimonte-Nelson HA, Acosta JI, Talboom JS (2010) Neuroscientists as cartographers: mapping the crossroads of gonadal hormones, memory and age using animal models. Molecules 15:6050–6105

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brett M, Baxendale S (2001) Motherhood and memory: a review. Psychoneuroendocrinology 26:339–362

Cardoso FL, Brites D, Brito MA (2011) Looking at the blood–brain barrier: molecular anatomy and possible investigation approaches. Brain Res Rev 64:328–363

Crozier A, Jaganath IB, Clifford MN (2009) Dietary phenolics: chemistry, bioavailability and effects on health. Nat Prod Rep 26:1001–1043

Cura AJ, Carruthers A (2010) Acute modulation of sugar transport in brain capillary endothelial cell cultures during activation of the metabolic stress pathway. J Biol Chem 285:15430–15439

Cura AJ, Carruthers A (2012) AMP kinase regulation of sugar transport in brain capillary endothelial cells during acute metabolic stress. Am J Physiol Cell Physiol 303:C806–C814

Duenas M, Surco-Laos F, Gonzalez-Manzano S, Gonzalez-Paramas AM, Santos-Buelga C (2011) Antioxidant properties of major metabolites of quercetin. Eur Food Res Technol 232:103–111

Faria A, Pestana D, Azevedo J, Martel F, de Freitas V, Azevedo I, Mateus N, Calhau C (2009) Absorption of anthocyanins through intestinal epithelial cells—putative involvement of GLUT2. Mol Nutr Food Res 53:1430–1437

Ferraris RP (2001) Dietary and developmental regulation of intestinal sugar transport. Biochem J 360:265–276

Fleegal MA, Hom S, Borg LK, Davis TP (2005) Activation of PKC modulates blood–brain barrier endothelial cell permeability changes induced by hypoxia and posthypoxic reoxygenation. Am J Physiol Heart Circ Physiol 289:H2012–H2019

Foy MR, Baudry M, Akopian GK, Thompson RF (2010) Regulation of hippocampal synaptic plasticity by estrogen and progesterone. Vitam Horm 82:219–239

Frolova A, Flessner L, Chi M, Kim ST, Foyouzi-Yousefi N, Moley KH (2009) Facilitative glucose transporter type 1 is differentially regulated by progesterone and estrogen in murine and human endometrial stromal cells. Endocrinology 150:1512–1520

Gerhart DZ, Leino RL, Borson ND, Taylor WE, Gronlund KM, McCall AL, Drewes LR (1995) Localization of glucose transporter GLUT 3 in brain: comparison of rodent and dog using species-specific carboxyl-terminal antisera. Neuroscience 66:237–246

Goncalves P, Araujo JR, Pinho MJ, Martel F (2009) Modulation of butyrate transport in Caco-2 cells. Naunyn Schmiedebergs Arch Pharmacol 379:325–336

Goncalves P, Araujo JR, Pinho MJ, Martel F (2011) In vitro studies on the inhibition of colon cancer by butyrate and polyphenolic compounds. Nutr Cancer 63:282–294

Gonzalez-Manzano S, Gonzalez-Paramas A, Santos-Buelga C, Duenas M (2009) Preparation and characterization of catechin sulfates, glucuronides, and methylethers with metabolic interest. J Agric Food Chem 57:1231–1238

Gutierrez-Merino C, Lopez-Sanchez C, Lagoa R, Samhan-Arias AK, Bueno C, Garcia-Martinez V (2011) Neuroprotective actions of flavonoids. Curr Med Chem 18:1195–1212

Henry JD, Rendell PG (2007) A review of the impact of pregnancy on memory function. J Clin Exp Neuropsychol 29:793–803

Ikeda K, Nagashima T, Wu S, Yamaguchi M, Tamaki N (1997) The role of calcium ion in anoxia/reoxygenation damage of cultured brain capillary endothelial cells. Acta Neurochir Suppl 70:4–7

Jiang X, Kenerson H, Aicher L, Miyaoka R, Eary J, Bissler J, Yeung RS (2008) The tuberous sclerosis complex regulates trafficking of glucose transporters and glucose uptake. Am J Pathol 172:1748–1756

Keating E, Goncalves P, Lemos C, Costa F, Campos I, Smith SB, Bridges CC, Martel F (2007) Progesterone inhibits folic acid transport in human trophoblasts. J Membr Biol 216:143–152

Keating E, Goncalves P, Campos I, Costa F, Martel F (2009) Folic acid uptake by the human syncytiotrophoblast: interference by pharmacotherapy, drugs of abuse and pathological conditions. Reprod Toxicol 28:511–520

Kiyatkin EA, Lenoir M (2012) Rapid fluctuations in extracellular brain glucose levels induced by natural arousing stimuli and intravenous cocaine: fueling the brain during neural activation. J Neurophysiol 108:1669–1684

Krikorian R, Shidler MD, Nash TA, Kalt W, Vinqvist-Tymchuk MR, Shukitt-Hale B, Joseph JA (2010) Blueberry supplementation improves memory in older adults. J Agric Food Chem 58:3996–4000

Lau FC, Shukitt-Hale B, Joseph JA (2006) Age-related neuronal and behavioral deficits are improved by polyphenol-rich blueberry supplementation. In: Luo Y, Packer L (eds) Oxidative stress and age-related neurodegeneration. CRC Press-Taylor and Francis, Boca Raton, pp 373–393

Maggio M, Dall'Aglio E, Lauretani F, Cattabiani C, Ceresini G, Caffarra P, Valenti G, Volpi R, Vignali A, Schiavi G, Ceda GP (2012) The hormonal pathway to cognitive impairment in older men. J Nutr Health Aging 16:40–54

Maher F, Vannucci SJ, Simpson IA (1994) Glucose transporter proteins in brain. FASEB J 8:1003–1011

Malhi GS, Tanious M, Das P, Berk M (2012) The science and practice of lithium therapy. Aust N Z J Psychiatr 46:192–211

Maraldi T, Rugolo M, Fiorentini D, Landi L, Hakim G (2006) Glucose transport activation in human hematopoietic cells M07e is modulated by cytosolic calcium and calmodulin. Cell Calcium 40:373–381

Martel F, Monteiro R, Calhau C (2010) Effect of polyphenols on the intestinal and placental transport of some bioactive compounds. Nutr Res Rev 23:47–64

Medina RA, Meneses AM, Vera JC, Guzman C, Nualart F, Rodriguez F, de los Angeles Garcia M, Kato S, Espinoza N, Monso C, Carvajal A, Pinto M, Owen GI (2004) Differential regulation of glucose transporter expression by estrogen and progesterone in Ishikawa endometrial cancer cells. J Endocrinol 182:467–478

Mullen W, Boitier A, Stewart AJ, Crozier A (2004) Flavonoid metabolites in human plasma and urine after the consumption of red onions: analysis by liquid chromatography with photodiode array and full scan tandem mass spectrometric detection. J Chromatogr A 1058:163–168

Ohtsuki S, Ikeda C, Uchida Y, Sakamoto Y, Miller F, Glacial F, Decleves X, Scherrmann JM, Couraud PO, Kubo Y, Tachikawa M, Terasaki T (2013) Quantitative targeted absolute proteomic analysis of transporters, receptors and junction proteins for validation of human cerebral microvascular endothelial cell line hCMEC/D3 as a human blood–brain barrier model. Mol Pharm 10:289–296

Owen L, Sunram-Lea SI (2011) Metabolic agents that enhance ATP can improve cognitive functioning: a review of the evidence for glucose, oxygen, pyruvate, creatine, and L-carnitine. Nutrients 3:735–755

Simpson IA, Carruthers A, Vannucci SJ (2007) Supply and demand in cerebral energy metabolism: the role of nutrient transporters. J Cereb Blood Flow Metab 27:1766–1791

Singh M, Arseneault M, Sanderson T, Murthy V, Ramassamy C (2008) Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. J Agric Food Chem 56:4855–4873

Spencer JP (2009a) Flavonoids and brain health: multiple effects underpinned by common mechanisms. Genes Nutr 4:243–250

Spencer JP (2009b) The impact of flavonoids on memory: physiological and molecular considerations. Chem Soc Rev 38:1152–1161

Spencer JP (2009c) Nutrients and brain health: an overview. Genes Nutr 4:225–226

Spencer JP, Vauzour D, Rendeiro C (2009) Flavonoids and cognition: the molecular mechanisms underlying their behavioural effects. Arch Biochem Biophys 492:1–9

Uchida Y, Ohtsuki S, Katsukura Y, Ikeda C, Suzuki T, Kamiie J, Terasaki T (2011) Quantitative targeted absolute proteomics of human blood–brain barrier transporters and receptors. J Neurochem 117:333–345

Verrotti A, D'Egidio C, Agostinelli S, Gobbi G (2012) Glut1 deficiency: when to suspect and how to diagnose? Eur J Paediatr Neurol 16:3–9

Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P, Male DK, Roux F, Greenwood J, Romero IA, Couraud PO (2005) Blood–brain barrier-specific properties of a human adult brain endothelial cell line. FASEB J 19:1872–1874

Youdim KA, Qaiser MZ, Begley DJ, Rice-Evans CA, Abbott NJ (2004) Flavonoid permeability across an in situ model of the blood–brain barrier. Free Radic Biol Med 36:592–604

CHAPTER III

“Flavonoids as dopaminergic neuromodulators"

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RESEARCH ARTICLE

Flavonoids as dopaminergic neuromodulators

Manuela Meireles[1], Eduardo Moura[2], Maria Augusta Vieira-Coelho[2], Celestino Santos-Buelga[3], Susana Gonzalez-Manzano[3], Montserrat Dueñas[3], Nuno Mateus[4], Ana Faria[1,4,5] and Conceição Calhau[1,6]

[1] Department of Biochemistry, Faculty of Medicine, University of Porto, Porto, Portugal
[2] Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Porto, Porto, Portugal
[3] Unidad de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno s/n, Salamanca, Espanha
[4] REQUIMTE/LAQV, Faculty of Sciences, University of Porto, Porto, Portugal
[5] Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal
[6] CINTESIS - Center for Research in Health Technologies and Information Systems, Faculty of Medicine, University of Porto, Porto, Portugal

Scope: The present study aimed to characterize and evaluate flavonoids effects on organic cation uptake in neuronal cells.
Methods and results: Uptake experiments were conducted using radiolabeled methyl-4-phenylpyridinuim ([^{3}H]-MPP$^+$), in human neuronal dopaminergic cells, SH-SY5Y. Catechin did not alter [^{3}H]-MPP$^+$ uptake, however its metabolite 4'-methyl-catechin decreased it by almost 50%. Epicatechin and its methylated metabolites also decreased [^{3}H]-MPP$^+$ uptake. Interestingly, the quercetin flavonol and its metabolite conjugated with glucuronic acid, as well as the flavanones naringenin and hesperitin, increased [^{3}H]-MPP$^+$ uptake.
Conclusion: These results showed that different classes of flavonoids, as well as its metabolites, differently influence neuronal organic cation uptake. Several xeno- and endobiotics, including neurotransmitters, are organic cations. Specific food recommendations may be beneficial in pathological conditions where levels of neurotransmitters, as dopamine, are either increased or decreased.

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1 Introduction

Flavonoids are a class of polyphenol compounds widely distributed in many fruits and vegetables. Besides their antioxidant, anti-inflammatory and other health benefits they are known to modulate several drugs or nutrients transport at intestinal [1–3], placental [3] and blood-brain barrier level [4]. Its potential in protect brain from neurodegeneration have been growing [5] with some epidemiological studies showing a positive association between flavonoids and total polyphenols intake and a better resistance to dementia, typical of aging [6, 7]. Interactions within intracellular signaling pathways, changes in vascular function and protection against neurotoxins and neuroinflammation are some of the mechanisms suggested for their action [8]. However, modulation of endobiotics transport at neuronal level may account as an important mechanism of flavonoids action.

Correspondence: Conceição Calhau
E-mail: ccalhau@med.up.pt

Various xeno- and endobiotics belong to the class of organic cations. Drugs from a wide array of therapeutic groups, including antihistamines, skeletal muscle relaxants, calcium channel blockers and ß-adrenoceptor blocking agents, are organic cations [9]. In addition, several endogenous bioactive amines (such as dopamine, catecholamines, 5-hydroxytryptamine and histamine) and some vitamins (such as thiamine and riboflavin) are also organic cations [9]. Being some of these molecules important neurotransmitters, modulation of organic cation transport in dopaminergic neurons may play a role in disorders like depression, bipolar disorder, Parkinson's disease (PD) and attention deficit hyperactivity disorder (ADHD) where it is known that neurotransmission is seriously affected [10].

The aim of this study was to understand the modulation of organic cations uptake in neuronal cells by flavonoids and its metabolites. With this purpose, methyl-4-phenylpyridinuim (MPP$^+$) uptake, a positively charged molecule broadly used as in vitro model for organic cation substrate in transport studies

[3], was characterized in SH-SY5Y cells and the involvement of some signaling pathways was explored.

2 Materials and methods

2.1 Reagents

[^{3}H]-1-methyl-4-phenylpyridinuim ([^{3}H]-MPP$^+$), specific activity 40–50 Ci/mmol (American Radiolabeled Chemicals Inc., St. Louis, E.U.A.), (+)-catechin, (-)-epicatechin, quercetin, naringenin, hesperitin, 4′,5,7-trihydroxyisoflavone (genistein), BAPTA-AM, calmidazolium, H-89 dihydrochloride hydrate, KN-62, corticosterone, penicillin G, amphotericin B, streptomycin, HEPES, clonidine, verapamil, cyclosporin A, U73122, lithium chloride, choline chloride, potassium chloride, trypsin-EDTA, (Sigma-Aldrich®, Madrid, Spain); PD 98058, SB 203580 and SP600125 (Research Biochemicals International, Natick, USA); dimethylsulfoxide (DMSO), triton X-100 (Merck®, Darmstadt, Germany).

3′-*O*-Methylcatechin and 3- and 4′-*O*-methylepicatechin were prepared by hemisynthesis as described [11]. Quercetin-3-*O*-glucuronide was isolated from green bean pods as previously reported [12].

2.2 Cell and culture conditions

The SH-SY5Y cell line is a human neuroblastoma cell line commonly used as in vitro model of dopaminergic neurons. They acquire a dopaminergic phenotype after a retinoic acid (RA) and phorbol12-myristate13-acetate (TPA) differentiation [13, 14]. The cell line was obtained from the American Type Culture Collection (ATCC, CRL-2266, Rockville, MD, USA) and was used between passage number 12–19. Cells were maintained in a humidified atmosphere of 5% CO_2–95% air and were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM-F12Ham, Sigma-Aldrich Chemicals®, Madrid, Spain), supplemented with 1.19 g/L $NaHCO_3$, 3.47 g/L HEPES, 5% fetal bovine serum, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (all from Sigma). Culture medium was changed every 2–3 days and the culture was split every 7 days. For subculturing, cells were removed enzymatically (0.25% trypsin–EDTA, 5 min, 37°C), split from 1:10 to 1:20 and subcultured in plastic dishes (Orange Scientific®, USA).

For transport studies cells were subcultured in plastic supports and 24 h after seeding, differentiated with RA 10 μM for three days, and TPA 80 nM for three more days [13].

2.3 Characterization and modulation of MPP$^+$ uptake in SH-SY5Y cells

Transport studies were performed in differentiated cells seven days after initial seed on plastic supports, [^{3}H]-MPP$^+$ being applied to the medium facing the apical cell membrane. Initially, the growth medium was aspirated and the cells were washed with buffer at 37°C, with the following composition (in mM): 140 NaCl, 5 KCl, 10 glucose, 10 HEPES, 1 $MgSO_4$, 2.5 $CaCl_2$ and pH 7.4. Buffer experiment was modified to test pH, sodium and potential dependency, by changing pH, replacing NaCl with LiCl or choline chloride or increasing KCl concentration, respectively. Then cells were preincubated with respective buffer for 30 min at 37°C. To study compounds effect they were present in both pre-incubation and incubation buffer. Uptake was initiated by the addition of 0.2 mL medium at 37°C with 200 nM [^{3}H]-MPP$^+$ and tested compounds. Incubation was stopped after 5 min by placing the cells on ice and rinsing them with 0.5 mL ice-cold buffer. Cells were then solubilized with 0.3 mL 0.1% (v/v) Triton X-100 (in 5 mM Tris–HCl, pH 7.4), at room temperature overnight. Radioactivity in cells was measured by liquid scintillation counting. The final protein content of cell monolayers was determined by Bradford's method [15], using human serum albumin as standard.

2.4 Statistical analysis

Values are expressed as the arithmetic mean ± SEM. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance (ANOVA) followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. Differences were considered significant when $p<0.05$.

3 Results

3.1 Characterization of methyl-4-phenylpyridinium ([^{3}H]-MPP$^+$) uptake by SH-SY5Y

Cellular uptake of [^{3}H]-methyl-4-phenylpyridinium ([^{3}H]-MPP$^+$) was determined using SH-SY5Y cells as a model of human dopaminergic neurons. It was verified that [^{3}H]-MPP$^+$ uptake in these cells occurs in a time-dependent way, and that [^{3}H]-MPP$^+$ uptake was linear with time for up to 8 min of incubation (data not shown). All subsequent experiments were performed using a 5 min incubation period, in the presence or absence of compounds, in order to determine initial rates of uptake.

Sodium dependence was analysed by replacement of sodium chloride (NaCl) for lithium chloride (LiCl) or choline chloride (ChoCl) in both pre-incubation and incubation media. No inhibition of [^{3}H]-MPP$^+$ uptake was observed suggesting that [^{3}H]-MPP$^+$ uptake in these cells is sodium-independent (Fig. 1A). However, replacement of NaCl by potassium chloride (KCl) significantly increased [^{3}H]-MPP$^+$ uptake, showing a dependence of potential in these uptake (Fig. 1B). Influence of pH on [^{3}H]-MPP$^+$ uptake was also studied and there were no significant changes in [^{3}H]-MPP$^+$ uptake with pH variations (data not shown).

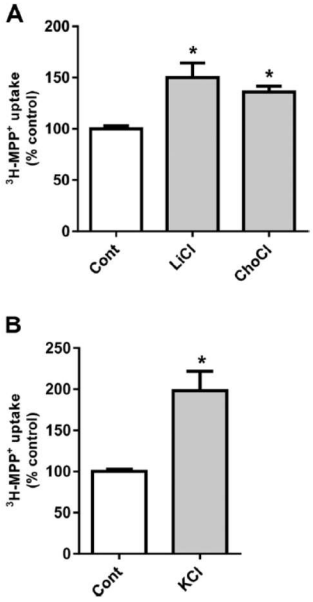

Figure 1. (A) Effect of Na^+ dependence on [^{3}H]-MPP^+ uptake by SH-SY5Y differentiated cells. NaCl in pre-incubation and incubation buffer was replaced by lithium chloride and choline chloride (ChoCl) to test Na^+ dependence. (B) Effect of potential on [^{3}H]-MPP^+ uptake by SH-SY5Y differentiated cells by replacement of NaCl in pre-incubation and incubation buffer by potassium chloride. Cells were pre-incubated for 30 min with respective buffer and then incubated at 37°C with buffer and [^{3}H]-MPP^+ (200 nM) for 5 min. Results are expressed as arithmetic means ± SEM ($n = 3$), $^*P < 0.05$ versus control.

3.2 Intracellular signalling pathways

In order to investigate which signaling pathways could be involved on [^{3}H]-MPP^+ uptake, SH-SY5Y cells were exposed to some specific inhibitors for 30 min (Table 1).

Involvement of Ca^{2+} intracellular on [^{3}H]-MPP^+ uptake was studied by testing the effect of Ca^{2+} chelator BAPTA-AM [16]. There was a significant increase on [^{3}H]-MPP^+ uptake in the presence of this compound. However, calmidazolium, a Ca^{2+}/calmodulin (CaM) inhibitor [17] did not have any effect on [^{3}H]-MPP^+ uptake. Inhibition of CaM-dependent protein kinase II (CaMKII) also increased [^{3}H]-MPP^+ uptake on SH-SY5Y cells producing similar effects as those of BAPTA-AM.

The involvement of mitogen activated protein kinases (MAPK) was investigated by testing the effect of specific inhibitors of MAP kinase ERK1/2 (PD 98059), p38 (SB 203580) and JNK (SP 600125) [18]. Inhibition of JNK induced a significant increase on [^{3}H]-MPP^+ uptake suggesting a role of this pathway on this uptake. None of other two compounds had significant effects.

Genistein, an inhibitor of protein tyrosine kinases (PTK) [19], and H89 [20], a selective inhibitor of protein kinase A (PKA), were used to test the involvement of protein kinase pathways. Genistein significantly increase [^{3}H]-MPP^+ uptake while H89 did not had any effect. Inhibition of phospholipase C (PLC) by U73122 [21], in turn, produces a significant decrease in [^{3}H]-MPP^+ uptake while cyclosporin A (a calcineurin inhibitor) did not have any effect.

Table 1. Effect of several signaling pathways inhibitors on [^{3}H]-MPP^+ uptake by SH-SY5Y differentiated cells

	[^{3}H]-MPP^+ uptake (% control)
BAPTA-AM 50 μM	152 ± 15*
Calmidazolium 25 μM	96 ± 5
KN-62 10 μM	163 ± 6*
H89 10 μM	97 ± 7
Genistein 10 μM	138 ± 8*
Cyclosporin A 1 μM	87 ± 6
U73122 30 μM	64 ± 3*
ERK ½ inhibitor 25 μM	109 ± 8
p38 inhibitor 25 μM	109 ± 12
JNK inhibitor 25 μM	130 ± 7*

Intracellular Ca^{2+} chelator (BAPTA-AM), CaM inhibitor (calmidazolium), CaMKII inhibitor (KN-62), PKA inhibitor (H89), PTK inhibitor (genistein), PLC inhibitor (U73122), calcineurin inhibitor (cyclosporin A) and inhibitors of MAPKs ERK1/2 (PD 98058), p38 (SB 203580) and JNK (SP 600125) were tested. Cells were preincubated 30 min with the tested compounds and then incubated at 37°C with the tested compounds and [^{3}H]-MPP^+ (200 nM) for 5 min. Results are expressed relatively as percentage of control (means ±SEM), $^*P < 0.05$ versus control.

3.3 Flavonoids and metabolites effect

The effect of different flavonoids and some of their metabolites on organic cation uptake in dopaminergic neurons was tested. Catechin did not induce significant alterations on [^{3}H]-MPP^+ uptake; however its metabolite 4′-methyl-catechin decreased it. Epicatechin was able to decrease [^{3}H]-MPP^+ uptake only at the higher concentration tested (100 μM). Curiously, a lower concentration of the 3′-methyl-epicatechin and 4′-methyl-epicatechin, two of its metabolites, had the same effect, significantly decreasing [^{3}H]-MPP^+ uptake (Fig. 2A).

Quercetin, one of the most widespread flavonoid in the human diet, at the higher concentration tested, increased [^{3}H]-MPP^+ uptake and quercetin-3-*O*-glucuronide, one of the main quercetin metabolite, also increased [^{3}H]-MPP^+ uptake at 30 μM (Fig. 2B).

Naringenin and hesperitin, two representative flavanones, increased [^{3}H]-MPP^+ uptake but this effect was not concentration dependent.

3.4 Transporters involved in [^{3}H]-MPP^+ uptake

To assess if Dopamine Transporter (DAT) was involved on [^{3}H]-MPP^+ uptake in dopaminergic neurons, a specific

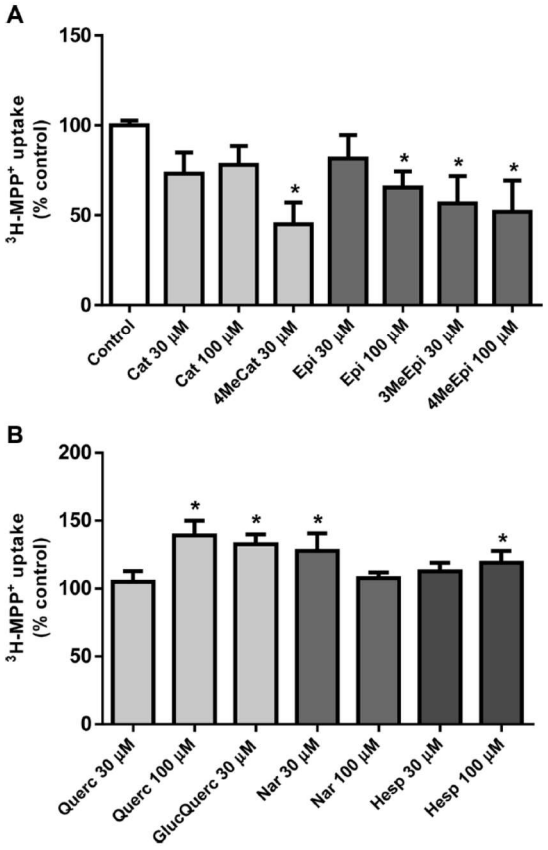

Figure 2. Effect of flavonoids and some of their metabolites on [^{3}H]-MPP$^+$ uptake by SH-SY5Y cells. Cells were preincubated for 30 min the tested compounds and then incubated at 37°C with [^{3}H]-MPP$^+$ (200 nM) for 5 min. (A) Effect of 30 and 100 µM of catechin and epicatechin and 30 µM of 4′-methyl-catechin, 4′-methyl-epicatechin and 30 µM of 3′-methyl-epicatechin on ^{3}H-MPP$^+$ uptake by SHSY5Y cells. (B) Effect of 30 and 100 µM of quercetin, naringenin and hesperitin and 30 µM of quercetin-3-*O*-glucuronide on [^{3}H]-MPP$^+$ uptake by SHSY5Y cells. Results are expressed as arithmetic means ± SEM ($n = 3$), *$P < 0.05$ versus control.

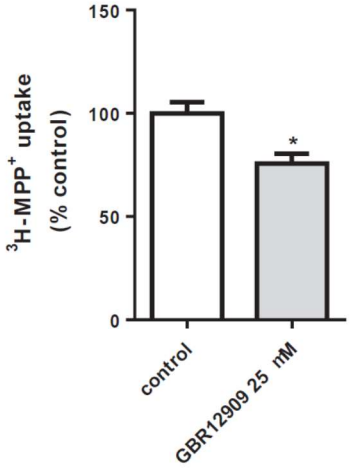

Figure 3. Effect of 25 µM of GBR12909 on [^{3}H]-MPP$^+$ uptake by differentiated SH-SY5Y cells. Cells were pre-incubated 30 min with the tested compound and then incubated at 37°C with [^{3}H]-MPP$^+$ (200 nM) for 5 min. Results are expressed as arithmetic means ± SEM ($n = 3$), *P<0.05 versus control.

inhibitor – GBR12909 (vanoxerine) – was tested [22]. Vanoxerine significantly decreased [^{3}H]-MPP$^+$ uptake demonstrating a relevant role of this transporter in this uptake (Fig. 3).

4 Discussion

Flavonoids are important components in the human diet that have been associated with health promoting effects [23–25]. Some attention has been recently given to these compounds as their consumption has been associated with the amelioration of several neuronal pathological conditions as well as memory loss and ageing [8, 26, 27]. Flavonoids are divided in different classes according to the level of oxidation and functional groups of C ring and its connection to B ring, while individual compounds within a class differ in the arrangements of hydroxyl, methoxyl and glycosidic side groups [22]. This chemical structure differences result not only in different physicochemical properties but, more importantly, in different biological activity [3, 28]. In addition, after ingestion flavonoids are subjected to metabolization which also changes the chemical structure of the compounds and consequently will have an impact in biological activity [22, 29]. Although permeability of blood-brain barrier to flavonoids has been demonstrated, the range of concentrations that could be in contact with neurons is still not clear [27, 30, 31].

In this study, different classes of flavonoids at different concentration were tested. The results obtained with the various flavonoids yielded different results. The flavan-3-ols catechin and epicatechin are stereoisomers, with very similar physicochemical properties, but results achieved with this two compounds are not the exactly the same, with epicatechin being a stronger inhibitor of [^{3}H]-MPP$^+$ uptake. The methylated forms of this compounds, one the most abundant forms circulating in vivo [32], showed a consistent inhibition of [^{3}H]-MPP$^+$ uptake that should be biologically relevant.

Despite the different models used, these results were in agreement with previous studies at intestinal level which also show an inhibitory effect on [^{3}H]-MPP$^+$ uptake by catechin [1]. This could indicate some similarity on the transporters used, on the interaction between substrate and flavonoid or on the intracellular signaling pathways involved on transporter regulation.

Interestingly, flavonoids belonging to other structural classes had opposite effects on [^{3}H]-MPP$^+$ uptake. While flavan-3-ols inhibited [^{3}H]-MPP$^+$ uptake, the flavonol quercetin, as well as its main metabolite, the glucuronic acid conjugated of quercetin, and also the flavanones naringenin and hesperitin increased this uptake. These different effects showed by different classes of flavonoids suggested structural-dependent interference.

The results presented suggested the involvement of Ca^{2+} pathways and of phosphorylation/dephosphorylation reactions on the [^{3}H]-MPP$^+$ uptake by SH-SY5Y. Phosphorylation/dephosphorylation is a common mechanism to regulate proteins, and membrane transporters are included in this class. Phospolipase C (PLC) seems to be directly involved, since its inhibition directly decrease the [^{3}H]-MPP$^+$ uptake.

Flavonoids also have shown ability to act on ionic channels and membrane conductivity [33] and particularly quercetin has potential to activate Ca^{2+} release [34]. Interference of flavonoids with Ca^{2+} release or Ca^{2+} signalization could be suggested as one of their mechanism of action. These results are consistent with the involvement of Ca^{2+} signalization in this transport activation. Also, inhibition of PLC is also associated with increase in intracellular [Ca^{2+}] [21]. Results presented in this work showed a determinant role of Ca^{2+} on [^{3}H]-MPP$^+$ uptake in this cell model. Moreover, KCl-induced depolarization and consequent influx of Ca^{2+} increased this uptake. The MAPK/JNK pathway and PTK seem to be also involved however the mechanisms remain unclear. Flavonoids, as anti-oxidants, may modify the redox environment modulating [^{3}H]-MPP$^+$ uptake. The redox changes has already been shown to influence [^{3}H]-MPP$^+$ uptake [1, 35]. Thus although not addressed in this study, changes in redox state may be consider to contribute to alterations in these transporters activity.

Through the use of the specific inhibitor GBR12909, it was confirmed an involvement of DAT in [^{3}H]-MPP$^+$ uptake in SH-SY5Y. These results are in agreement with previous studies that demonstrate the need of dopamine transporter on MPP$^+$ effect in dopaminergic neurons [36, 37]. The dopamine transporter belongs to a large family of Na^+ and Cl^- dependent transporters [38]. However, this work results' were not in accordance with this dependence, suggesting that more than one transporter are involved in this uptake. Yet, specific inhibition of OCT1, OCT3, SERT and NAT did not produce any effect on [^{3}H]-MPP$^+$ uptake (data not shown). Depression, bipolar disorder, PD and attention deficit hyperactivity disorder (ADHD) are associated with abnormal DA levels and DAT may be implicated in some of these conditions. Inhibitors or agonists of DAT are being considered as new approaches to treat these conditions [10]. This work results' showed a partial involvement of DAT on [^{3}H]-MPP$^+$ uptake, and therefore, a possible involvement of flavonoids on modulation of this transporter. DAT is responsible for the removal of dopamine from the synaptic cleft. Inhibition of this uptake by flavan-3-ols can affect neurotransmission by increasing dopamine on the level of the synaptic cleft. However naringenin, hesperitin, quercetin and metabolites increased this uptake. This increase can be helpful in psychoses where levels of dopamine are increased on synaptic cleft as schizophrenia or situations of drugs abuse like methamphetamines and cocaine [39, 40].

Overall, this work showed that organic cations uptake in dopaminergic neurons may be modulated by diet flavonoids and most interesting, by its biological metabolites, in structural-dependent way. This uptake seems to be regulated by Ca^{2+} pathways, phosphorylation/dephosphorylation mechanisms and have a partial involvement of DAT. These results suggested a possible interference of flavonoids with neurotransmitters transport, namely dopamine reuptake which may be relevant to explain the potential neuroprotective actions of some flavonoids.

A well-balanced diet, rich in vegetables and fruits where chocolate, tea and grapes are good sources of catechin and epicatechin, citrus fruit have abundant amounts of flavonones and onions and apples are rich sources of quercetin, may be helpful in restoring homeostasis in different dopamine disorders. Thus, despite the care that should be taken in extrapolating in vitro results, this study suggested that flavan-3-ols rich food could be helpful in PD while flavonols and flavonones sources could be more useful in people with psychoses or drug addiction.

MM and AF conducted the experiments, analyzed the data and draft the manuscript; EM designed and establish the cell differentiation protocol; CSB, SGM, MB and NM were responsible for flavonoids and metabolites synthesis and revised the manuscript; MAVC interpreted data and revised the article. AF and CC conceived and designed the study and revised the article.

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5 References

[1] Faria, A., Mateus, N., de Freitas, V., Calhau, C., Modulation., of MPP+ uptake by procyanidins in Caco-2 cells: involvement of oxidation/reduction reactions. *FEBS Lett.* 2006, *580*, 155–160.

[2] Monteiro, R., Calhau, C., Martel, F., Faria, A. et al., Modulation of MPP+ uptake by tea and some of its components in Caco-2 cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 2005, *372*, 147–152.

[3] Martel, F., Monteiro, R., Calhau, C., Effect of polyphenols on the intestinal and placental transport of some bioactive compounds. *Nutr. Res. Rev.* 2010, *23*, 47–64.

[4] Meireles, M., Martel, F., Araujo, J., Santos-Buelga, C. et al. Characterization and modulation of glucose uptake in a human blood-brain barrier model. *J. Memb Biol.* 2013, *246*, 669–677.

[5] Youdim, K. A., Spencer, J. P., Schroeter, H., Rice-Evans, C., Dietary flavonoids as potential neuroprotectants. *Biol. Chem.* 2002, *383*, 503–519.

[6] Kesse-Guyot, E., Fezeu, L., Andreeva, V. A., Touvier, M. et al., Total and specific polyphenol intakes in midlife are associated with cognitive function measured 13 years later. *J. Nutr.* 2012, *142*, 76–83.

[7] Letenneur, L., Proust-Lima, C., Le Gouge, A., Dartigues, J. F., Barberger-Gateau, P., Flavonoid intake and cognitive decline over a 10-year period. *Am. J. Epidemiol.* 2007, *165*, 1364–1371.

[8] Williams, R. J., Spencer, J. P., Flavonoids, cognition, and dementia: actions, mechanisms, and potential therapeutic utility for Alzheimer disease. *Free Radic. Biol. Med.* 2012, *52*, 35–45.

[9] Koepsell, H., Lips, K., Volk, C., Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharmaceutical Res.* 2007, *24*, 1227–1251.

[10] Vaughan, R. A., Foster, J. D., Mechanisms of dopamine transporter regulation in normal and disease states. *Trends Pharmacological Sci.* 2013, *34*, 489–496.

[11] Gonzalez-Manzano, S., Gonzalez-Paramas, A., Santos-Buelga, C., Duenas, M., Preparation and characterization of catechin sulfates, glucuronides, and methylethers with metabolic interest. *J. Agric. Food Chem.* 2009, *57*, 1231–1238.

[12] Dueñas, M., Mingo-Chornet, H., Pérez-Alonso, J. J., Di Paola-Naranjo, R. et al., Preparation of quercetin glucuronides and characterization by HPLC–DAD–ESI/MS. *Eur. Food Res. Technol.* 2008, *227*, 1069–1076.

[13] Presgraves, S. P., Ahmed, T., Borwege, S., Joyce, J. N., Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. *Neurotoxicity Res.* 2004, *5*, 579–598.

[14] Xie, H. R., Hu, L. S., Li, G. Y., SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. *Chin. Med. J. (Engl.)* 2010, *123*, 1086–1092.

[15] Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, *72*, 248–254.

[16] Collatz, M. B., Rudel, R., Brinkmeier, H., Intracellular calcium chelator BAPTA protects cells against toxic calcium overload but also alters physiological calcium responses. *Cell Calcium.* 1997, *21*, 453–459.

[17] Sampaio-Maia, B., Soares-da-Silva, P., Ca2+/calmodulin mediated pathways regulate the uptake of L-DOPA in mouse neuroblastoma neuro 2A cells. *Life Sci.* 2000, *67*, 3209–3220.

[18] Newton, R., Cambridge, L., Hart, L. A., Stevens, D. A. et al., The MAP kinase inhibitors, PD098059, UO126 and SB203580, inhibit IL-1beta-dependent PGE(2) release via mechanistically distinct processes. *Br. J. Pharmacol.* 2000, *130*, 1353–1361.

[19] Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H. et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 1987, *262*, 5592–5595.

[20] Bain, J., Plater, L., Elliott, M., Shpiro, N. et al. The selectivity of protein kinase inhibitors: a further update. *Biochem. J.* 2007, *408*, 297–315.

[21] Mogami, H., Lloyd Mills, C., Gallacher, D. V., Phospholipase C inhibitor, U73122, releases intracellular Ca2+, potentiates Ins(1,4,5)P3-mediated Ca2+ release and directly activates ion channels in mouse pancreatic acinar cells. *Biochem. J.* 1997, *324*, 645–651.

[22] Beecher, G. R., Overview of dietary flavonoids: nomenclature, occurrence and intake. *J. Nutr.* 2003, *133*, 3248S–3254S.

[23] Hollman, P. C., Katan M. B., Dietary flavonoids: intake, health effects and bioavailability. *Food Chem. Toxicol.* 1999, *37*, 937–942.

[24] Kaur, C., Kapoor, H. C., Antioxidants in fruits and vegetables - the millennium's health. *Int. J. Food Sci. Technol.* 2001, *36*, 703–725.

[25] Van Duyn, M. A., Pivonka, E., Overview of the health benefits of fruit and vegetable consumption for the dietetics professional: selected literature. *J. Am. Diet Assoc.* 2000, *100*, 1511–1521.

[26] Andrade, J. P., Assuncao, M., Protective effects of chronic green tea consumption on Age-related neurodegeneration. *Curr. Pharm. Des.* 2012, *18*, 4–14.

[27] Andres-Lacueva, C., Shukitt-Hale, B., Galli, R. L., Jauregui, O. et al., Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. *Nutr. Neurosci.* 2005, *8*, 111–120.

[28] Rice-Evans, C. A., Miller, N. J., Paganga, G., Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* 1996, *20*, 933–956.

[29] Duenas, M., Gonzalez-Manzano, S., Gonzalez-Paramas, A., Santos-Buelga, C., Antioxidant evaluation of O-methylated metabolites of catechin, epicatechin and quercetin. *J. Pharm. Biomed. Anal.* 2010, *51*, 443–449.

[30] Faria, A., Meireles, M., Fernandes, I., Santos-Buelga, C. et al. Flavonoid metabolites transport across a human BBB model. *Food Chem.* 2014, *149*, 190–196.

[31] Nehlig, A., The neuroprotective effects of cocoa flavanol and its influence on cognitive performance. *Br. J. Clin. Pharmacol.* 2013, *75*, 716–727.

[32] Okushio, K., Suzuki, M., Matsumoto, N., Nanjo, F., Hara, Y., Identification of (-)-epicatechin metabolites and their metabolic fate in the rat. *Drug Metab. Disposition* 1999, *27*, 309–316.

[33] Hendrich, A. B., Flavonoid-membrane interactions: possible consequences for biological effects of some polyphenolic compounds. *Acta Pharmacologica Sinica* 2006, *27*, 27–40.

[34] Lee, E. H., Meissner, G., Kim, D. H., Effects of quercetin on single Ca(2+) release channel behavior of skeletal muscle. *Biophys. J.* 2002, *82*, 1266–1277.

[35] Faria, A., Monteiro, R., Pestana, D., Freitas, V. et al. Intestinal oxidative state can alter nutrient and drug bioavailability. *Oxidative Med. Cell. Longevity* 2009, *2*, 322–327.

[36] Pifl, C., Giros, B., Caron, M. G., Dopamine transporter expression confers cytotoxicity to low doses of the parkinsonism-inducing neurotoxin 1-methyl-4-phenylpyridinium. *J. Neurosci.* 1993, *13*, 4246–4253.

[37] Bezard, E., Gross, C. E., Fournier, M. C., Dovero, S. et al., Absence of MPTP-induced neuronal death in mice lacking the dopamine transporter. *Exp. Neurol.* 1999, *155*, 268–273.

[38] Storch, A., Ludolph, A. C., Schwarz, J., Dopamine transporter: involvement in selective dopaminergic neurotoxicity and degeneration. *J. Neural. Transm.* 2004, *111*, 1267–1286.

[39] Deik, A., Saunders-Pullman, R., Luciano, M. S., Substance abuse and movement disorders: complex interactions and comorbidities. *Curr. Drug Abuse Rev.* 2012, *5*, 243–53.

[40] Sekine, Y., Iyo, M., Ouchi, Y., Matsunaga, T. et al. Methamphetamine-related psychiatric symptoms and reduced brain dopamine transporters studied with PET. *Am. J. Psychiatry* 2001, *158*, 1206–1214.

CHAPTER IV

"Effect of chronic consumption of blackberry extract on high-fat induced obesity and its correlation with metabolic and brain outcomes"

Food & Function

PAPER

Effect of chronic consumption of blackberry extract on high-fat induced obesity in rats and its correlation with metabolic and brain outcomes

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ManuelaMeireles,[a] Luís M. Rodríguez-Alcalá,[b] Cláudia Marques,[a] Sónia Norberto,[a] Joana Freitas,[a] Iva Fernandes,[c] Nuno Mateus,[c] Ana Gomes,[b] Ana Faria[a,c,d] and Conceição Calhau*[a,e]

Flavonoids have been presented as potential protectors against metabolic and cognitive dysfunction. However, mechanisms underlying these 'claims' have not been sufficiently explored. To analyse the effect of long-term supplementation with blackberry extract (BE) in the context of a high-fat or a standard diet, Wistar rats were divided into 4 groups (n = 6) fed with a standard or a high-fat diet, with or without BE supplementation at 25 mg per kg body weight per day. A high-fat diet significantly impaired glucose tolerance and increased body weight, caloric ingestion, very-low-density lipoprotein, triglycerides and cholesterol. Furthermore, it was observed that a high-fat diet increased dopamine content in the prefrontal cortex and decreased brain derived neurotrophic factor (BDNF) levels both in the prefrontal cortex and in plasma. BE supplementation only affected some of these aspects. BE slightly improved glucose metabolism and significantly decreased levels of lactate, independent of diet. BE decreased levels of BDNF and also interacted with the dopaminergic system, increasing dopamine turnover in the striatum, and reverting dopamine content induced by a high-fat diet in the prefrontal cortex. This study shows that, despite some particular benefits of anthocyanin supplementation, some long-term effects may not be desirable and further studies are needed to optimize ingestion conditions.

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1. Introduction

Obesity is a health problem that has reached epidemic proportions. In some European countries, more than one quarter of the population are already obese, presenting a body mass index (BMI) of greater than 30 kg m^{-2}.[1] This phenomenon is of equal or larger proportions in several other industrialized countries, *i.e.* in USA the incidence of obesity reached 35% in 2011–2012.[2] Overall, more than 60% of the US and European adult population are obese or overweight, with BMI > 25 kg m^{-2}.[3] This epidemic represents an economic and social burden since several co-morbidities are associated with obesity.[4] People with obesity have, among other features, an increased risk of suffering from diabetes, hypertension and cardiovascular diseases, cancer, and neurodegenerative diseases.[5] In the past decade, much attention has been paid to the potential negative effects of obesity on brain function and general risk of dementia.[3,6] Like obesity, dementia is also becoming a worldwide problem and an international priority, with millions of new cases every year.[7] As obesity and its co-morbidities are associated with brain dysfunction, the development of therapeutic strategies to treat one condition can have worthy improvements in the other.

Diet is an undeniable modifiable factor which may influence both the risk of obesity and impaired brain function. The exacerbation of both conditions by westernized diets, typically rich in fat and sugar, is well documented. *In vivo* studies have also shown that animals fed high-fat diets have several brain disorders, among them dysfunction of the dopaminergic system and impairment of neurogenesis.[8–10]

Fruits and vegetables, in contrast to high-fat and high-sugar diets, have been proven to reduce the risk of obesity and associated co-morbidities.[11,12] In addition, intake of fruits and

[a]Departamento de Bioquímica, Faculdade de Medicina, Universidade do Porto, Al. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal. E-mail: ccalhau@med.up.pt; Fax: +351 22 551 3624; Tel: +351 22 551 3624
[b]Centro de Biotecnologia e Química Fina (CBQF), Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal
[c]REQUIMTE, Laboratório Associado de Química Verde, Faculdade de Ciências, Universidade do Porto, 4169-009 Porto, Portugal
[d]Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
[e]Centro de Investigação em Tecnologias e Sistemas de Informação em Saúde (CINTESIS), Universidade do Porto, Al. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

vegetables has been associated with a decreased risk of cognitive decline or Parkinson's disease, in part due to their high content of natural polyphenols, including flavonoids.[13,14] In recent years, berries have attracted interest as a nutritional approach to improve health, metabolic and cognitive outcomes.[15–17] Blackberries, in particular, are naturally rich in flavonoids, specifically in anthocyanins, and although blackberry extracts have already shown positive effects in improving cognitive function and antioxidant status in animal studies,[18–21] there remains a need to further explore and clarify the full potential of anthocyanins.

This study aimed to explore the beneficial potential of long-term supplementation with blackberry anthocyanin extract in the context of a standard or a high-fat diet. For this purpose male Wistar rats fed with a standard or a high-fat diet for 17 weeks were supplemented with blackberry anthocyanin extract (25 mg per kg b.w.) and metabolic evolution as well as biomarkers of brain function were analysed.

2. Methods

2.1. Animal care and study design

Twenty-four male Wistar rats (200–250 g body weight; 7 weeks) were acquired from Harlan Laboratories (Santiga, Spain) and after two weeks of acclimatization were divided into four groups (n = 6 rats per group): (C) standard diet; (BE) standard diet + blackberry extract; (HF) high-fat diet; (HFBE) high-fat diet + blackberry extract. Animals were fed *ad libitum* with commercial diets: standard chow (4% fat) from Harlan (2014S, Teklad Diets, Harlan Laboratories, Santiga, Spain) and high-fat chow (45% fat) from Research Diet D12451 (New Brunswick, NJ, USA) for 17 weeks. Blackberry extract dose was weekly weight adjusted (25 mg per kg body weight), dissolved daily in sterile water and embedded in food pellets that animals had daily access to. Animals were maintained at 23–25 °C with a 12/12 h light–dark cycle, and housed two per cage. Food ingestion was measured twice a week. Animal handling and housing protocols followed European Union guidelines (86/609/EEC) and the Portuguese Act (129/92) for the use of experimental animals. The study obtained ethical approval from the Ethical Committee of the Faculty of Medicine, University of Porto and the São João Hospital Center.

2.2. Preparation of blackberry extract

Preparation of blackberry anthocyanin extract was achieved using previously described methods.[22,23] Briefly, blackberries (*Rubus fruticosus*) were extracted with 50% aqueous ethanol (pH 1.5, acidified with HCl) for 24 hours at 22 °C. The solution obtained was filtered (50 μm nylon membrane) and concentrated using a rotary evaporator under reduced pressure at 30 °C. The concentrated extract was added to a polyamide gel column (mesh 100–120) to remove sugars. The sugar-free anthocyanin extract was freeze-dried and stored at −20 °C. The anthocyanin extract was analyzed by HPLC at 520 nm, as previously described.[24] Following preparative HPLC, the purified extract was characterised for anthocyanin content by analytical HPLC coupled to UV-Vis, DAD-ESI/MS and NMR techniques.

2.3. Insulin tolerance test and glucose tolerance test

Insulin and glucose tolerance tests were performed at weeks 3, 8 and 15 of the treatment period with one day intervals between tests. For the insulin tolerance test, after 5 hours of food deprivation, a solution of insulin (0.5 U per kg weight) was administered by intraperitoneal injection.[25] Glucose levels were measured from tail blood with a Precision Xceed® glucometer before (0) and 30, 60, 90 and 120 minutes after insulin injection. The glucose tolerance test was performed after 5 hours of food deprivation; a solution of glucose (2 g per kg weight) was administered by oral gavage as previously described.[26] Glucose levels were measured before (0) and 15, 30, 60, 90 and 120 minutes after glucose administration.

2.4. Blood pressure measurements

Systolic blood pressure was measured with a tail cuff method.[27] To minimize stress-induced variations in blood pressure all measurements were taken in a peaceful environment and after two trials had been performed in the previous week to allow animals to become accustomed to the procedure. Values were registered when consistent between three consecutive measurements, and when the pulse was stable. This procedure took place at weeks 9 and 14 of the treatment period.

2.5. Tissue collection and body composition measurements

At the end of 17 weeks animals were anesthetized with ketamine + xylazine (50 mg kg^{-1} + 1 mg kg^{-1}), nasoanal lengths and abdominal circumference were obtained using a measuring tape, and the body composition of each rat was determined by bioelectrical impedance (quantum/S bioelectrical impedance analyser, RJL Systems, Akern SRL, Florence, Italy), according to the procedure described in the literature.[28] Animals were maintained with isoflurane during blood collection from the left ventricle. Blood was collected with heparinised needles and PMSF (phenylmethylsulfonyl fluoride), a protease inhibitor, was added at a final concentration of 100 μM. After centrifugation at 2000g for 15 min, plasma was collected and stored at −80 °C. Rats were decapitated for brain removal. Prefrontal cortex, striatum, the remaining brain, liver, subcutaneous adipose tissue (scAT) and mesenteric adipose tissue (mAT) were collected, immediately frozen with liquid nitrogen and stored at −80 °C until use. A small portion of scAT and mAT from each animal was also fixed in 10% buffered formaldehyde.

2.6. Biochemical analysis

For urine collection, rats were placed in metabolic cages, after being acclimated during the previous week. Routine plasma and urine biochemical analyses were performed in a certified Clinical Analysis Laboratory. In addition, adiponectin, leptin and brain derived neurotrophic factor (BDNF) were quantified using the following enzyme linked immunoabsorbent assay (ELISA) commercial kits: adiponectin (Life Technologies Ltd,

Paisley, UK), leptin (Merck Millipore, Madrid, Spain) and chemiKine brain derived neurotrophic factor (Merck Millipore, Madrid, Spain). For BDNF determination, a small portion of the whole brain was homogenized using 100 mM Tris/HCl, pH 7, containing 2% bovine serum albumin (BSA), 1 M NaCl, 4 mM EDTA·Na_2, 2% Triton X-100, 0.1% sodium azide and a protease inhibitor (cOmplete mini, Roche Diagnostics, USA). Homogenates were prepared using 4 μL of the described buffer for mg of tissue weight. All following procedures in plasma and brain homogenate were performed according to the manufacturers' instructions.

2.7. Morphometric analysis of adipose tissue

Following at least 48 h (4 °C) of formaldehyde fixation, the adipose tissues were dehydrated and finally embedded in paraffin. All samples were coded for a blind analysis and 3 μm-thick sections were obtained with a Leica® Microtome (RM2125RT, Lisbon, Portugal), and stained with haematoxylin and eosin to assess morphology. Digital images were acquired with a fluorescence microscope (Nikon Eclipse 50i®, Melville, USA), at a magnification of ×200 from randomly-selected different optical fields. Adipocyte area measurement was performed in 100 random adipocytes, using ImageJ Software[®] (National Institute of Health, Bethesda, USA).

2.9. Fatty acid analysis

For analysis of the total fatty acid (FA) composition, 200 mg of liver and 30 mg of adipose tissue were accurately weighed and prepared as previously described.[29] For quantification purposes, samples were mixed with 100 μL of tritridecanoin (1.34 mg ml^{-1}), used as an internal standard prior to derivatization. Fatty acid methyl esters (FAME) were analyzed in a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (50 m × 0.32 mm × 0.25 μm; SGE Europe Ltd, Courtaboeuf, France). Analysis conditions were as follows: injector (split 10 : 1; injection volume 1 μl) and detector temperatures were 250 °C and 270 °C respectively, carrier gas was hydrogen (11 psi) and the oven temperature program was started at 60 °C (held for 2 min) raised by 10 °C min^{-1} to 135 °C (held for 2 min), then raised by 10 °C min^{-1} to 165 °C (held for 2 min) and finally raised by 10 °C min^{-1} to 230 °C (held for 7 min). Supelco 37 and CRM-164 were used for the identification of fatty acids. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.079 μg FA ml^{-1}; LOQ: 0.264 μg FA ml^{-1}).

2.10. Catecholamine determination

The quantification of dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in striatum was performed by high pressure liquid chromatography with electrochemical detection (HPLC-ED), as previously described.[30] In brief, aliquots of 1.5 ml of 0.2 M perchloric acid in which tissues were kept were placed in 5 ml conical-based glass vials with 50 mg alumina, and the pH of the samples was immediately adjusted to 8.6 with Tris buffer. The adsorbed catecholamines were then eluted from the alumina with 200 μL of 0.2 M perchloric acid and centrifuged at 1200*g* in Spin-X tubes with 0.22 μm pore membrane (Costar®, Tewksbury MA, EUA); 50 μL of the eluant was injected into HPLC (Gilson Medical Electronics, Villiers, le Bel, France) and 3,4-dihydroxybenzylamine was used as an internal standard. The results were adjusted for tissue weight.

2.11. Statistical analysis

All the groups were tested for the effects of diet (D), treatment (BE) and their interaction (D × BE) by two-way ANOVA using GraphPad Prism® 6.0 Software. When interaction between diet and treatment were significantly different, means were compared using Fisher's LSD multiple-comparison post-test. Correlations were tested using the Pearson correlation test. Statistical significance was considered when $p < 0.05$.

3. Results

3.1. Body weight, weight gain and caloric ingestion

All animals started the treatment at similar weights (246.5 ± 1.9 g), and at end of the treatment animals maintained similar weights between groups C and BE (429 ± 28 g and 433 ± 29 g, respectively). The body weights of animals from HF and HFBE were 551 ± 68 g and 581 ± 63 g, respectively. The increase in body weight over time was more pronounced in groups fed the HF diet (Fig. 1A). Weight gain was significantly increased by diet (Fig. 1B). Effect of supplementation with BE on body weight gain was not significant.

Food ingestion was converted from g to kCal, knowing that standard and high-fat chow have 2.9 kCal and 4.73 kCal per gram, respectively. As show in Fig. 1C, caloric ingestion was affected by both diet and by BE supplementation (Fig. 1C). Curiously, there was a significant interaction between the effects of type of diet and BE supplementation on caloric ingestion. Caloric ingestion of animals supplemented with BE on the HF diet was slightly higher (82 kCal per animal per day) than those not supplemented (75 kCal per animal per day), however it was not reflected in weight gain.

3.2. Metabolic parameters

Glucose and insulin tolerance tests were performed at weeks 3, 8 and 15 of the treatment. HF diet impaired the overall glucose sensibility of the animals, seen as an increase of total area under the curve (AUC) (Fig. 2A). This effect was seen as early as after three weeks of study, and it was maintained until the end of treatment (Fig. 2B–D). Animals from the BE group presented a tendency to a lower AUC, significant at the end of the test on week 8.

After 15 weeks of treatment, both animals' basal blood glucose and glucose tolerance response were significantly affected by diet but not by BE supplementation (Fig. 2D).

Glycaemic levels in insulin tolerance test was affected by diet after only three weeks of treatment (Fig. 3A). BE supplementation significantly increase insulin response at

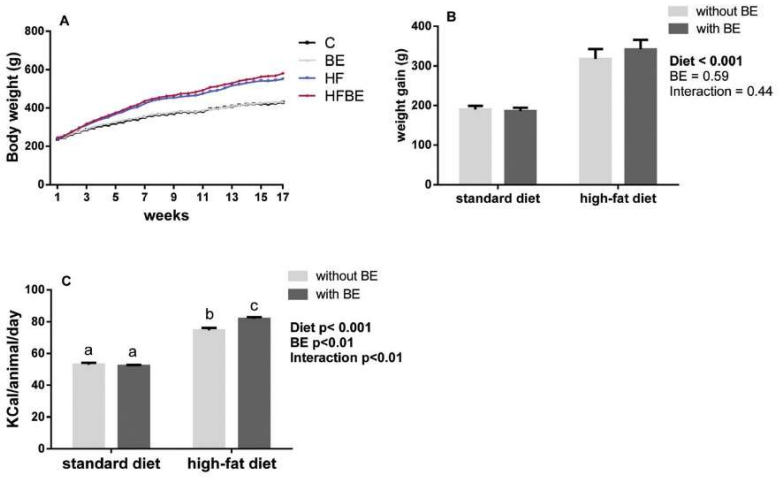

Fig. 1 Effects of blackberry anthocyanin extract (BE) on weight of Wistar rats fed a standard or a high-fat diet over 17 weeks, expressed as weight evolution during the treatment time (A) or weight gain at the end of 17 weeks (B) and effects on food ingestion (C). C – standard diet fed group; BE – standard diet supplemented with blackberry anthocyanin extract fed group; HF – high-fat diet fed group; HFBE – high-fat diet supplemented with blackberry anthocyanin extract group (n = 6). Results are expressed as mean ± SEM. Statistical significance was considered when $p < 0.05$.

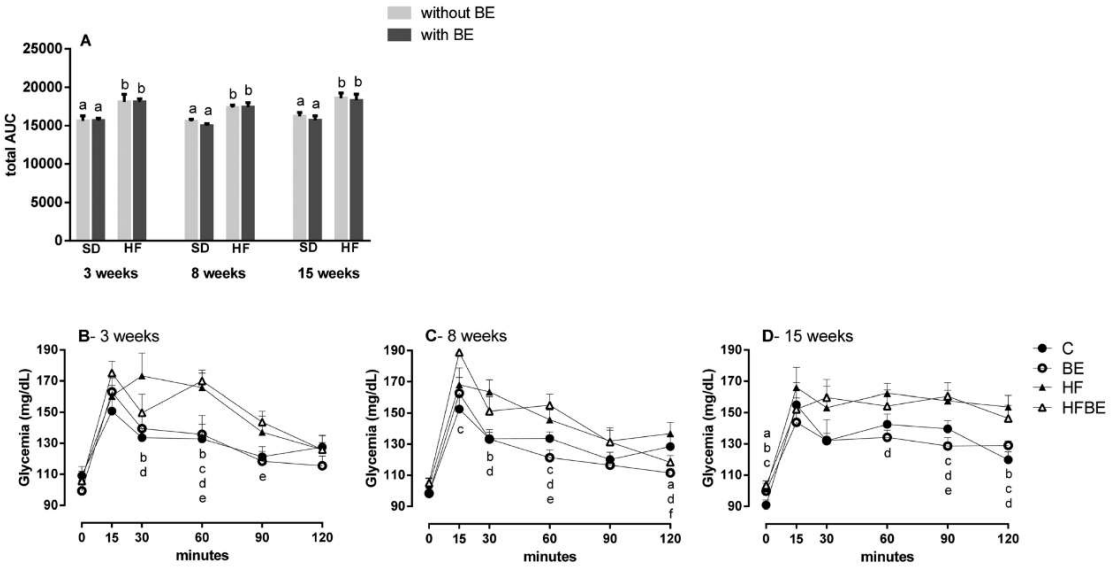

Fig. 2 Effects of blackberry anthocyanin extract (BE) on glycaemic response to oral glucose test in Wistar rats fed a standard or a high-fat diet. (A) Area under the curve of total glycaemic response over 120 min following the oral gavage of a solution of glucose (2 g per kg weight). (B)–(D) represent the glycaemic response at each time point during the 120 min, measured at 3, 8 and 15 weeks of treatment. C – standard diet fed group; BE – standard diet supplemented with blackberry anthocyanin extract fed group; HF – high-fat diet fed group; HFBE – high-fat diet supplemented with blackberry anthocyanin extract group (n = 6). Letters were presented at a specific point every time the following differences were seen (a) C *vs.* BE; (b) C *vs.* HF; (c) C *vs.* HFBE; (d) BE *vs.* HF; (e) BE *vs.* HFBE; (f) HF *vs.* HFBE. Statistical significance was tested by two-way ANOVA considering time and diet as factors, followed by Fisher's LSD test. Different superscript letters represent statistical differences between means ($p < 0.05$).

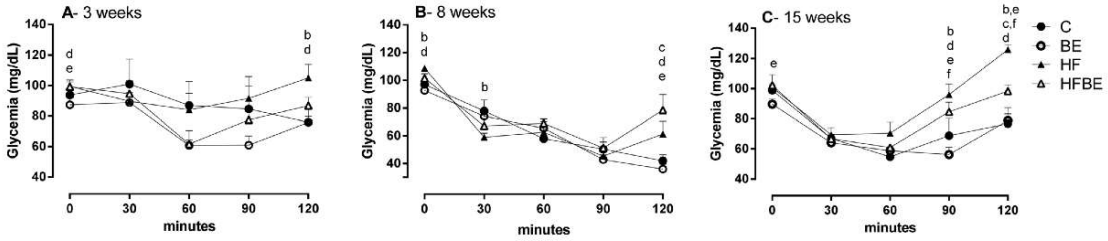

Fig. 3 Effects of blackberry anthocyanin extract (BE) on glycaemic response to insulin tolerance test in Wistar rats fed a standard or a high-fat diet after (A) 3 weeks; (B) 8 weeks and (C) 15 weeks. C – standard diet fed group; BE – standard diet supplemented with blackberry anthocyanin extract fed group; HF – high-fat diet fed group; HFBE – high-fat diet supplemented with blackberry anthocyanin extract group (n = 6). Letters were presented at a specific point every time the following differences were seen (a) C *vs.* BE; (b) C *vs.* HF; (c) C *vs.* HFBE; (d) BE *vs.* HF; (e) BE *vs.* HFBE; (f) HF *vs.* HFBE. Statistical significance was tested by two-way ANOVA considering time and diet as factors, followed by Fisher's LSD test. Different superscript letters represent statistical differences between means ($p < 0.05$).

Table 1 Systolic blood pressure (SBP) at a middle and end stage of the study design

	C	BE	HF	HFBE	D	BE	D × BE
SBP – 9 weeks	137.5 (11.2)	129.6 (5.7)	145.2 (9.8)	139.7 (14.5)	0.058	0.15	0.79
SBP – 15 weeks	129.9 (14.3)	133.3 (9.0)	137.3 (13.4)	135.9 (20.5)	0.42	0.86	0.69

SBP – systolic blood pressure. Standard diet (C); standard diet + blackberry anthocyanin extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanin extract (HFBE). Values are expressed as mean (SD). The significance of diet (D), blackberry extract (BE) or interaction between both factors (D × BE) were tested by two-way ANOVA and expressed as p values.

60 min. Curiously, at week 8 (Fig. 3B) animals fed with the HF diet had a higher response to insulin, presenting a lower peak at 30 min; however the animals fed with the standard diet were the ones that sustained the decrease in glycaemia as seen at 120 min. As can be seen in Fig. 3C, after 15 weeks, animals from the HFBE group had a better response to insulin than those from the HF group.

Systolic blood pressure seemed to be affected by diet but not by BE supplementation after 8 weeks of treatment. No effect was seen after 14 weeks of treatment (Table 1).

3.3. Biochemical parameters

At the end of treatment plasmatic biochemical analyses were performed. As described in Table 2, the HF diet affected lipid metabolism, increasing very low density lipoprotein (VLDL), triglycerides (TG) and cholesterol levels of the animals. Regarding hepatic enzymes, only alkaline phosphatase (ALP) activity was strongly increased by the HF diet. Also, levels of urea were increased by the HF diet. BE supplementation decreased aspartate aminotransferase activity (ASAT) and decreased creatinine kinase activity (CK). Plasmatic lactate levels were strongly affected by BE supplementation, but not with the high-fat diet. Interaction between diet and BE supplementation affected albumin and creatinine levels. While in C animals albumin seemed to decrease and creatinine increase, the opposite effect was seen in animals fed with the HF diet.

Renal function was affected by the HF diet: there was an increase in urine excretion of urea, sodium and albumin and a decrease in glycosuria. BE also contributed to an increase in renal excretion of urea.

Plasmatic levels of brain derived neurotropic factor (BDNF) were significantly decreased in the HF fed animals. The BE group showed decreased levels, but the supplementation had no effect regarding HF fed groups (Table 3). BDNF levels in the brain frontal cortex were measured to correlate with plasmatic ones. Cortical BDNF levels were decreased both by diet and by BE supplementation. There was not a correlation between plasmatic and central BDNF (r = 0.361 p = 1.129) considering all tested animals. However, when considering each group isolated, control groups (C) had a strong association between both localizations (r = −0.967 p = 0.033) but both HF diet and BE supplementation disrupted this correlation.

3.4. Adipose tissue distribution, adipokine levels, and fatty acid composition

HF diet was the main contributing factor to variance in nasoanal length, waist circumference, free fatty mass, fatty mass weight and percentage, leading to an increase in all these parameters (Table 4). The plasmatic levels of adipokines adiponectin and leptin (Fig. 4A and B) were both increased by HF diet. BE supplementation also contributed to an increase in animals' leptin levels but not in adiponectin levels. The ratio between the pro-inflammatory leptin and the anti-inflammatory adiponectin showed that there is still an increase induced by the HF diet but no effect of BE (Fig. 4C).

Table 2 Biochemical parameters in plasma and urine samples

	C	BE	HF	HFBE	D	BE	D × BE
Plasma analyses							
Cholesterol (mg dL^{-1})	103.8 (16.1)	100.0 (19.7)	105.6 (17.2)	133.2 (13.6)	**<0.05**	0.14	0.05
HDL (mg dL^{-1})	80.8 (14.9)	88.3 (16.7)	75.0 (12.8)	89.2 (13.8)	0.72	0.14	0.64
VLDL (mg dL^{-1})	30.0 (9.5)	26.3 (2.5)	37.2 (5.9)	36.3 (5.3)	**<0.01**	0.45	0.64
TG (mg dL^{-1})	150.2 (47.6)	131.5 (11.8)	185.0 (29.8)	181.3 (27.2)	**<0.01**	0.45	0.62
ASAT (U L^{-1} at 37 °C)	163.6 (67.1)	101.0 (31.0)	168.6 (41.6)	117.3 (25.0)	0.61	**<0.05**	0.78
ALAT (U L^{-1} at 37 °C)	47.2 (9.2)	51.5 (11.9)	41.4 (8.2)	41.2 (4.5)	0.07	0.60	0.63
ALP (U L^{-1} at 37 °C)	79.7 (11.6)	92.8 (5.8)	132.0 (29.8)	135.2 (17.0)	**<0.001**	0.33	0.55
Proteins (g dL^{-1})	6.08 (0.35)	6.00 (0.22)	6.16 (0.19)	6.47 (0.33)	0.05	0.41	0.15
Albumin (g dL^{-1})	3.67 (0.2)^{a,b}	3.37 (0.2)^{a}	3.52 (0.2)^{a,b}	3.77 (0.3)^{b}	0.26	0.81	**<0.05**
Creatinine (mg dL^{-1})	0.40 (0.10)^{a}	0.53 (0.02)^{b}	0.52 (0.05)^{b}	0.44 (0.11)^{a,b}	0.62	0.54	**<0.01**
CK (U L^{-1} at 37 °C)	1087 (358)	443 (189)	783 (236)	485 (155)	0.27	**<0.001**	0.15
Urea (mg dL^{-1})	30.2 (1.7)	32.2 (2.6)	36.6 (4.5)	40.2 (5.8)	**<0.001**	0.14	0.67
Uric acid (mg dL^{-1})	0.83 (0.17)	0.74 (0.14)	0.92 (0.23)	0.93 (0.31)	0.20	0.71	0.62
Iron (μg dL^{-1})	291.0 (35.3)	271.8 (37.6)	259.4 (32.2)	268.2 (14.8)	0.29	0.75	0.40
Sodium (mmol L^{-1})	140.8 (4.6)	142.5 (3.3)	136.0 (1.6)	141.5 (4.7)	0.14	0.06	0.32
Potassium (mmol L^{-1})	5.50 (0.75)	5.55 (0.53)	5.78 (0.61)	6.00 (0.82)	0.29	0.69	0.80
Chlorides (mmol L^{-1})	103.2 (3.7)^{a,b}	100.8 (1.6)^{a}	100.2 (3.3)^{a}	107.8 (8.0)^{b}	0.40	0.27	**<0.05**
Calcium (mg dL^{-1})	9.9 (0.97)	10.7 (0.30)	10.7 (0.45)	11.1 (0.59)	0.07	0.07	0.60
Phosphorus (mg dL^{-1})	10.3 (0.71)	10.2 (1.25)	10.3 (0.37)	10.8 (0.66)	0.40	0.67	0.46
Magnesium (mg dL^{-1})	2.66 (0.16)	2.66 (0.16)	2.32 (0.23)	2.20 (0.17)	**<0.001**	0.40	0.36
Lactate (mg dL^{-1})	7.85 (1.94)	5.58 (1.00)	7.90 (1.85)	3.80 (1.82)	0.25	**<0.001**	0.23
Urine analyses							
Total urine (24 h)	17.3 (6.0)^{a,b}	12.7 (5.5)^{a}	12.5 (6.5)^{a}	22.5 (7.1)^{b}	0.38	0.35	**<0.05**
Glucose (mg dL^{-1})	14.8 (1.3)	19.0 (3.7)	10.2 (1.5)	11.2 (0.7)	**<0.001**	0.08	0.09
Urea 24 h (g per day)	0.17 (0.03)	0.19 (0.05)	0.24 (0.03)	0.35 (0.11)	**<0.01**	**<0.05**	0.14
Sodium (mmol per day)	0.53 (0.26)	0.62 (0.31)	0.70 (0.14)	0.93 (0.25)	**<0.05**	0.17	0.51
Potassium (mmol per day)	1.78 (0.36)	2.02 (0.71)	1.95 (0.35)	2.58 (0.62)	0.14	0.08	0.41
Microalbuminuria (mg per day)	0.04 (0.04)	0.05 (0.02)	0.16 (0.13)	0.12 (0.07)	**<0.05**	0.50	0.82

Standard diet (C); standard diet + blackberry anthocyanin extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanin extract (HFBE); HDL – high density lipoprotein; VLDL – very low density lipoprotein; TG – triglycerides; ASAT – aspartate aminotransferase; ALAT – alanine aminotransferase; ALP – alkaline phosphatase; CK – creatinine kinase. Values are expressed as mean (SD). The significance of diet (D), blackberry extract (BE) or interaction between both factors (D × BE) were tested by two-way ANOVA and expressed as p values. Post-hoc Fishers's LSD test was performed when interaction between factors was present. Mean values with different superscript letters are significantly different ($p < 0.05$).

Table 3 Brain derived neurotropic factor (BDNF) levels in plasma and brain homogenates

	C	BE	HF	HFBE	D	BE	D × BE
BDNF plasmatic (pg mL^{-1})	18.84 (4.0)^{a}	12.16 (3.7)^{b}	8.07 (2.1)^{b}	8.289 (3.7)^{b}	<0.001	0.06	<0.05
BDNF brain (pg per mg tissue)	0.88 (0.15)^{a}	0.57 (0.10)^{b}	0.68 (0.10)^{b}	0.57 (0.04)^{b}	<0.05	<0.001	<0.05

Standard diet (C); standard diet + blackberry anthocyanins extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanin extract (HFBE); values are expressed as mean (SD). The significance of diet (D), blackberry extract (BE) or interaction between both factors (D × BE) were tested by two-way ANOVA, expressed as p values, and followed by Fishers's LSD test. Mean values with different superscript letters are significantly different ($p < 0.05$).

Table 4 Body dimension and composition

	C	BE	HF	HFBE	D	BE	D × BE
Nasoanal length (cm)	25.2 (0.18)	25.0 (0.58)	26.7 (1.14)	27.1 (0.82)	<0.0001	0.752	0.348
Waist circumference (cm)	18.3 (0.87)	18.6 (0.83)	21.1 (1.10)	21.7 (1.25)	<0.0001	0.296	0.724
Free fatty mass (g)	246.6 (11.5)	248.3 (11.0)	295.1 (27.5)	307.3 (26.1)	<0.0001	0.417	0.539
Fatty mass (g)	181.2 (16.6)	183.9 (16.9)	259.2 (41.2)	278.3 (40.0)	<0.0001	0.400	0.525

Standard diet (C); standard diet + blackberry anthocyanins extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanins extract (HFBE). Values are expressed as mean (SD). The significance of diet (D), blackberry extract (BE) or interaction between both factors (D × BE) were tested by two-way ANOVA and expressed as p values.

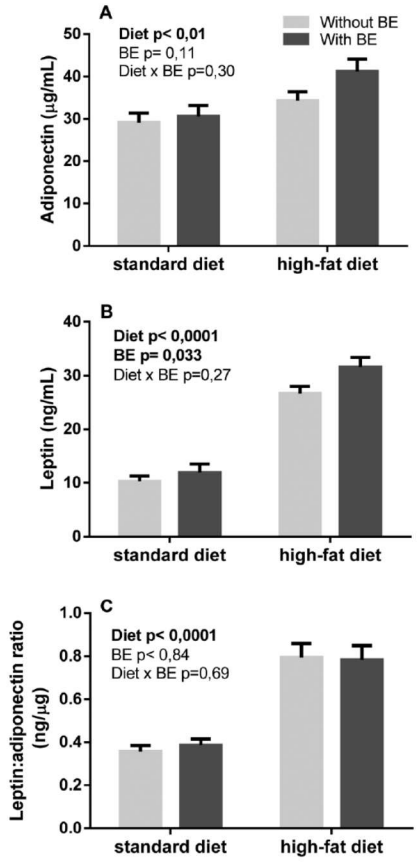

Fig. 4 Effects of blackberry anthocyanin extract (BE) on the release of adipokines on Wistar rats fed a standard or high-fat diet for 17 weeks (n = 6). Results are expressed as mean ± SEM. Statistical significance was tested by two-way ANOVA considering diet and blackberry supplementation as factors, followed by Fisher's LSD test. Statistical significance was considered when $p < 0.05$.

The morphology of adipose tissue was only affected by type of diet (Fig. 5A). Surprisingly, the HF diet increased adipocyte area only in subcutaneous depots (Fig. 5A). Fatty acid composition was analysed in the liver and on two distinct deposits of adipose tissue, known to have different metabolic implications: the subcutaneous and mesenteric adipose tissue. The concentrations of each fatty acid are described in Tables 5–9. Compared with C animals, HF diet animals showed a greater accumulation of total monounsaturated fatty acids (MUFA) in mesenteric adipose tissue, fewer polyunsaturated fatty acids (PUFA) (Table 5) and no differences in total saturated fatty acids (SFA) (Table 6). This effect was mainly due to greater accumulation of fatty acids as the monounsaturated oleic acid (C18:1 c9) and less as polyunsaturated linoleic acid (C18:2 c9c12). In subcutaneous adipose tissue (Tables 7 and 8), the HF diet increased SFA and MUFA with no effect on PUFA. BE supplementation did not have an effect on the global SFA, MUFA or PUFA. Nevertheless, some differences were observed in particular fatty acids within these classes, specifically in C17i and C15:1 c10, both increased in the mAT of the BE group; also C17i was increased in the scAT of these animals. There were no significant effects of BE on fatty acid composition in the liver (Table 9).

3.5. Dopamine content

Dopamine (DA) levels were quantified in the prefrontal cortex and the striatum. DA levels were affected by type of diet in the prefrontal cortex and there was an interaction of BE extract with this effect. As can be seen in Fig. 6A, although not having an effect on C animals, supplementation with BE reverted the increase in DA content seen in HF animals. In the striatum, type of diet did not affect DA levels while BE supplementation significantly decreased DA content (Fig. 6B). Levels of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were not affected by any factor (Fig. 6C). This reflected an increase in DOPAC/DA ratio, indicating a higher DA turnover with BE supplementation (Fig. 6D).

4. Discussion

This study aimed to analyse the impact of long-term BE anthocyanin consumption with a global metabolic approach in rats with or without diet-induced obesity. Several metabolic parameters were analysed: resistance to insulin, glucose tolerance,

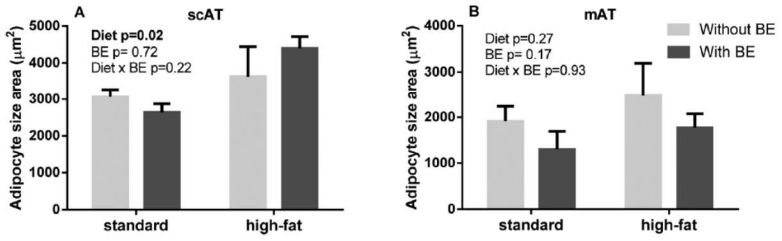

Fig. 5 Effects of blackberry anthocyanin extract (BE) on the adipocyte area of mesenteric adipose tissue (mAT) (A) and subcutaneous adipose tissue (scAT) (B) of Wistar rats fed a standard or a high-fat diet for 17 weeks (n = 6). Results are expressed as mean ± SEM. Statistical significance was considered when $p < 0.05$.

Table 5 Saturated fatty acid composition (μg FA per mg tissue) in mesenteric adipose tissue (MAD) samples

	C	BE	HF	HFBE
SFA	205.56 (33.31)	208.37 (29.45)	212.44 (35.87)	233.19 (27.53)
C12	0.32 (0.08)	0.29 (0.06)	0.29 (0.03)	0.35 (0.06)
C14	6.39^{a} (1.34)	5.97^{a} (1.23)	4.63^{b} (0.36)	$5.61^{a,b}$ (0.53)
C15 ai	0.07^{a} (0.03)	0.07^{a} (0.02)	0.03^{b} (0.00)	0.04^{b} (0.00)
C15	2.02^{a} (0.37)	1.99^{a} (0.21)	0.89^{b} (0.11)	0.99^{b} (0.06)
C16	170.20^{a} (27.52)	170.35^{a} (26.99)	142.96^{b} (21.97)	$161.19^{a,b}$ (15.16)
C17i	0.52^{a} (0.14)	0.75^{b} (0.13)	0.35^{b} (0.07)	0.40^{b} (0.05)
C17 ai	0.54^{a} (0.13)	0.55^{a} (0.14)	0.42^{b} (0.07)	0.48^{b} (0.06)
C17	1.70^{a} (0.37)	1.79^{a} (0.22)	2.52^{b} (0.47)	2.62^{b} (0.41)
C18 i	1.13^{a} (0.29)	1.47^{a} (0.23)	0.72^{b} (0.20)	0.74^{b} (0.19)
C18	20.44^{a} (4.53)	22.59^{a} (3.16)	57.96^{b} (13.41)	59.09^{b} (12.53)
C20	0.70 (0.24)	0.80 (0.17)	0.81 (0.23)	0.75 (0.27)
C21	0.07^{a} (0.02)	0.09^{a} (0.02)	0.05^{b} (0.02)	0.05^{b} (0.01)
C22	0.23^{a} (0.09)	0.27^{a} (0.07)	0.18^{b} (0.06)	0.17^{b} (0.06)
C23	$0.05^{a,b}$ (0.01)	0.06^{b} (0.01)	0.04^{a} (0.01)	0.04^{a} (0.00)
C24	1.18^{a} (0.30)	1.33^{a} (0.15)	0.57^{b} (0.13)	0.68^{b} (0.12)

ai: branched chain fatty acid, anteiso; i: branched chain fatty acid, iso. Standard diet (C); standard diet + blackberry anthocyanin extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanin extract (HFBE). Values are expressed as mean (SD). ^{a,b,c,d} Superscript letters in a row for significant differences among groups ($p < 0.05$) after one-way ANOVA followed by Bonferroni adjustment.

Table 6 Unsaturated fatty acid composition (μg FA per mg tissue) in mesenteric adipose tissue (MAD) samples

	C	BE	HF	HFBE
MUFA	235.25^{a} (35.89)	242.47^{a} (30.67)	331.81^{b} (50.51)	353.60^{b} (36.72)
C14:1	0.39^{a} (0.15)	0.37^{a} (0.12)	0.10^{b} (0.01)	0.13^{b} (0.04)
C15:1 c10	0.62^{a} (0.15)	0.76^{a} (0.13)	0.34^{b} (0.10)	0.38^{b} (0.07)
C16:1 t9	0.13^{a} (0.04)	0.13^{a} (0.02)	0.22^{b} (0.03)	0.25^{b} (0.02)
C16:1 c7	2.78^{a} (0.38)	2.65^{a} (0.38)	3.56^{b} (0.52)	3.79^{b} (0.33)
C16:1 c9	24.14^{a} (7.77)	22.47^{a} (8.71)	7.29^{b} (1.00)	10.00^{b} (2.47)
C16:1 c11	0.33^{a} (0.09)	0.28^{a} (0.08)	0.12^{b} (0.02)	0.16^{b} (0.02)
C17:1 c9	0.20^{a} (0.03)	0.19^{a} (0.03)	0.15^{b} (0.02)	$0.17^{a,b}$ (0.02)
C17:1 c10	1.08^{a} (0.24)	1.04^{a} (0.21)	1.26^{b} (0.15)	1.48^{b} (0.12)
C18:1 t	0.87^{a} (0.33)	0.95^{a} (0.24)	2.48^{b} (0.40)	2.47^{b} (0.30)
C18:1 c9	167.98^{a} (25.95)	174.29^{a} (18.91)	290.91^{b} (45.08)	307.25^{b} (34.37)
C18:1 c11	30.29^{a} (4.53)	32.11^{a} (4.34)	20.01^{b} (3.08)	21.89^{b} (2.19)
C18:1 c12	0.13^{a} (0.05)	0.14^{a} (0.05)	0.38^{b} (0.03)	0.41^{b} (0.03)
C18:1 c13	0.57^{a} (0.11)	0.56^{a} (0.09)	0.44^{b} (0.06)	$0.54^{a,b}$ (0.06)
C18:1 t16	0.48 (0.13)	0.55 (0.13)	0.51 (0.09)	0.53 (0.09)
C20:1 c9	2.02^{a} (0.44)	2.32^{a} (0.34)	3.19^{b} (0.78)	3.21^{b} (0.63)
C20:1 c11	3.20^{a} (0.76)	3.57^{a} (0.59)	0.88^{b} (0.24)	0.99^{b} (0.21)
C22:1 c9	0.11^{a} (0.05)	$0.15^{a,b}$ (0.03)	0.08^{b} (0.03)	0.08^{b} (0.02)
C24:1	0.10^{a} (0.05)	0.11^{a} (0.03)	0.06^{b} (0.01)	0.05^{b} (0.02)
PUFA	245.70^{a} (30.41)	249.96^{a} (30.35)	181.19^{b} (15.12)	194.72^{b} (12.22)
C18:2 t9t12	0.17 (0.04)	0.17 (0.04)	0.15 (0.02)	0.18 (0.02)
C18:2 c9t12	0.06^{a} (0.02)	0.05^{a} (0.01)	0.09^{b} (0.02)	0.12^{b} (0.02)
C18:2 t9c12	0.57^{a} (0.11)	0.53^{a} (0.11)	0.27^{b} (0.04)	0.30^{b} (0.06)
C18:2 c9c12	228.73^{a} (28.13)	233.44^{a} (28.34)	168.69^{b} (14.20)	179.88^{b} (11.73)
C18:3 t9t12c15	0.34 (0.06)	0.37 (0.04)	0.36 (0.06)	0.37 (0.05)
C18:3 c6c9c12	0.83^{a} (0.16)	$0.75^{a,b}$ (0.11)	0.64^{b} (0.08)	0.76^{b} (0.05)
C18:3 c9t12t15	0.37 (0.09)	0.45 (0.07)	0.43 (0.07)	0.45 (0.08)
C18:3 c9c12c15	7.64^{a} (1.77)	7.37^{a} (1.64)	4.80^{b} (0.53)	5.83^{b} (0.99)
C18:2 c9t11	0.22^{a} (0.09)	0.24^{a} (0.08)	0.63^{b} (0.06)	0.70^{b} (0.06)
C20:2 c11c14	1.16^{a} (0.28)	1.27^{a} (0.16)	2.08^{b} (0.45)	2.37^{b} (0.36)
C20:3 c8c11c14	0.65 (0.21)	0.64 (0.09	0.52 (0.08)	0.66 (0.09)
C20:4 AA	3.19^{a} (1.08)	2.98^{a} (0.37)	1.65^{b} (0.23)	2.05^{b} (0.35)
C20:3 c11c14c17	0.11^{a} (0.04)	0.10^{a} (0.01)	0.19^{b} (0.03)	0.23^{b} (0.05)
C20:5 n3	0.18^{a} (0.04)	0.16^{a} (0.03)	0.08^{b} (0.01)	0.09^{b} (0.02)
C22:2 c13c16	0.14^{a} (0.04)	0.13^{a} (0.02)	0.09^{b} (0.02)	0.08^{b} (0.02)
C22:5 n6	0.39^{a} (0.16)	0.41^{a} (0.08)	0.16^{b} (0.03)	0.18^{b} (0.04)
C22:5 n3	0.42^{a} (0.16)	0.44^{a} (0.13)	0.20^{b} (0.04)	0.23^{b} (0.06)
C22:6 DHA	0.54^{a} (0.29)	0.46^{a} (0.08)	0.18^{b} (0.04)	0.25^{b} (0.07)
μg mg^{-1}	686.51 (92.98)	700.80 (81.61)	725.44 (98.09)	781.51 (70.09)

c: *cis* double bond; t: *trans* double bond; AA: arachidonic acid; n3: omega 3 fatty acid; n6: omega 6 fatty acid; DHA: docosahexanoic fatty acid. Standard diet (C); standard diet + blackberry anthocyanin extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanin extract (HFBE). Values are expressed as mean (SD). ^{a,b,c,d} Superscript letters in a row for significant differences among groups ($p < 0.05$) after one-way ANOVA followed by Bonferroni adjustment.

Table 7 Saturated fatty acid composition (μg FA per mg tissue) in subcutaneous adipose tissue (SAD) samples

	C	BE	HF	HFBE
SFA	212.75^{a,b} (29.98)	249.04^{a,b} (22.50)	263.69^{b} (41.84)	285.47^{b} (46.28)
C12	0.49 (0.14)	0.45 (0.11)	0.55 (0.12)	0.51 (0.10)
C14	7.44 (0.91)	7.75 (1.82)	7.69 (1.33)	7.86 (1.14)
C15 ai	0.08^{a} (0.03)	0.08^{a} (0.02)	0.06^{b} (0.01)	0.05^{b} (0.01)
C15	2.25^{a} (0.45)	2.57^{a} (0.34)	1.32^{b} (0.21)	1.36^{b} (0.18)
C16	176.36 (24.19)	204.10 (18.35)	185.34 (29.67)	201.54 (30.12)
C17i	0.47^{a} (0.14)	0.82^{b} (0.14)	0.47^{a} (0.05)	0.52^{a} (0.09)
C17 ai	0.54 (0.10)	0.61 (0.10)	0.60 (0.09)	0.65 (0.09)
C17	1.73^{a} (0.45)	2.20^{a} (0.42)	2.92^{b} (0.47)	3.20^{b} (0.45)
C18i	1.03^{a} (0.15)	1.48^{a} (0.20)	0.80^{b} (0.07)	0.91^{b} (0.23)
C18	20.10^{a} (5.20)	26.15^{a} (5.26)	61.63^{b} (10.49)	66.43^{b} (15.68)
C20	0.49^{a} (0.13)	0.64^{a,b} (0.15)	0.63^{a,b} (0.13)	0.74^{b} (0.20)
C21	0.07 (0.02)	0.10 (0.01)	0.05 (0.02)	0.12 (0.21)
C22	0.16 (0.04)	0.22 (0.04)	0.14 (0.02)	0.16 (0.03)
C23	0.06 (0.01)	0.08 (0.03)	0.07 (0.01)	0.07 (0.02)
C24	1.48^{a,b} (0.30)	1.80^{a} (0.21)	1.41^{a,b} (0.31)	1.37^{b} (0.19)

ai: branched chain fatty acid, anteiso; i: branched chain fatty acid, iso. Standard diet (C); standard diet + blackberry anthocyanin extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanin extract (HFBE). Values are expressed as mean (SD). ^{a,b,c,d} Superscript letters in a row for significant differences among groups ($p < 0.05$) after one-way ANOVA followed by Bonferroni adjustment.

blood pressure, biochemical parameters and adipose tissue morphology, function and fatty acid storage.

Wistar rats were supplemented with an anthocyanin BE in a normal and in an obesity context for 17 weeks. The HF diet insult resulted, as expected, in overweight animals and some metabolic dysfunctions such as impairment in glucose tolerance and insulinemic response, increase in percentage of fat body weight, increase in scAT adipocyte size and increase in adipokine production. Although after 9 weeks of treatment, systolic blood pressure seemed altered, hypertension was not observed at the end.

As diet seems to be a key factor in controlling and counteracting the induction of obesity and associated co-morbidities, an extract from blackberry, rich in anthocyanins, was simultaneously given to a group of animals. This supplementation seemed to induce a better glycaemic response in both BE and HFBE animals. Some studies have shown a decrease in glycaemic response after anthocyanin consumption. The mechanisms behind this effect are not completely clear, but it is possible that it involves an effect on glucose transport.[31] Cyanidin-3-glucoside and delphinidin-3-glucoside have been associated with anti-diabetic properties in different *in vitro* and *in vivo* models.[32] Obesity has an associated low-grade chronic inflammation component, and it is still unclear whether it is a cause or a consequence of associated co-morbidities. An increase in adipocyte size, for instance, prompts adipose tissue inflammation, which is also dependent on the type of fatty acids stored.[33] Adipocyte size has been shown to be an indirect measurement of inflammation as it correlates with several risk factors of metabolic dysfunction.[33] The propensity to lower adipocyte size in mesenteric tissue may be predictive of a decreased risk of inflammation by BE supplementation. Localization of adipose tissue can predict the risk of metabolic consequences, more associated with visceral fat, while subcutaneous fat can have a protective effect.[34] Our results show that type of diet significantly increases cholesterol levels, but curiously this effect was only visible on the HFBE group, and not on the HF group, as would be expected from previous literature.[35,36] This effect may be, in part, due to the decrease in HDL seen in the HF groups and prevented in the HFBE group.

A very unusual but relevant result was the reduction of lactate release to the bloodstream after BE consumption. Hyperlactatemia has been found in obese humans[37,38] and previous studies have shown that, besides muscle, lactate can also be produced in other tissues including adipose tissue.[39] These lower lactate levels in groups with anthocyanin BE extract consumption may be seen as positive, since lactate levels are co-related with cardiovascular diseases and overall mortality.[40]

BE intake decreased BDNF content in both the plasma and the brain of standard-fed animals, which is in agreement with the results of Klein *et al.*, who have shown that blood BDNF concentrations correlate positively with BDNF levels in the hippocampus of rats and pigs.[41] It is well documented that BDNF is involved in synaptic plasticity, neuronal differentiation and survival of neurons, and thus its increase is usually associated with beneficial outcomes. Nevertheless, Miyazaki *et al.*[42] observed significant increases in serum BDNF levels in patients with neurodevelopmental disorders, such as autism or mental retardation, compared to normal controls. Also, Si-Hoon demonstrated that plasma BDNF levels have a significant positive correlation with the severity of inattention symptoms in children.[43] While El-Gharbawy *et al.*[44] showed in 2006 that plasmatic BDNF concentrations were decreased in obese individuals, a recent study has also shown that obesity does not affect BDNF levels.[45] Low levels of BDNF have been associated with changes in dopamine receptors,[46] and by contrast, increasing levels of BDNF were found to increase dopamine turnover.[47,48] BDNF gene expression in the frontal cortex of DAT knockout mice was shown to be reduced.[49] Both the HF

Table 8 Unsaturated fatty acid composition (μg FA per mg tissue) in subcutaneous adipose tissue (SAD) samples

	C	BE	HF	HFBE
MUFA	249.77^{a} (32.29)	281.16^{a} (29.12)	407.67^{b} (60.69)	449.86^{b} (61.37)
C14:1	0.44^{a} (0.06)	0.45^{a} (0.12)	0.21^{b} (0.04)	0.22^{b} (0.05)
C15:1 c10	0.67^{a} (0.13)	0.98^{b} (0.16)	0.50^{c} (0.05)	0.52^{c} (0.11)
C16:1 t9	0.17^{a} (0.03)	0.16^{a} (0.03)	0.33^{b} (0.06)	0.35^{b} (0.04)
C16:1 c7	3.20^{a} (0.47)	3.35^{a} (0.41)	4.65^{b} (0.82)	4.80^{b} (0.59)
C16:1 c9	28.26^{a} (7.49)	27.48^{a} (6.84)	15.68^{b} (2.53)	18.55^{b} (5.64)
C16:1 c11	0.39^{a} (0.07)	0.38^{a} (0.08)	0.21^{b} (0.05)	0.21^{b} (0.03)
C17:1 c9	0.21 (0.03)	0.24 (0.04)	0.21 (0.04)	0.21 (0.03)
C17:1 c10	1.26^{a} (0.23)	1.34^{a} (0.17)	1.88^{b} (0.27)	2.10^{b} (0.29)
C18:1 t	1.10^{a} (0.27)	1.19^{a} (0.34)	3.30^{b} (0.51)	3.33^{b} (0.46)
C18:1 c9	176.73^{a} (22.18)	201.44^{a} (22.99)	348.64^{b} (52.56)	383.85^{b} (53.61)
C18:1 c11	31.49^{a,b} (4.43)	37.04^{a} (4.08)	25.13^{c} (3.82)	28.02^{c} (3.76)
C18:1 c12	0.15^{a} (0.05)	0.15^{a} (0.03)	0.51^{b} (0.07)	0.54^{b} (0.07)
C18:1 c13	0.60 (0.11)	0.68 (0.07)	0.68 (0.09)	0.75 (0.11)
C18:1 t16	0.42^{a} (0.08)	0.48^{a} (0.08)	0.61^{b} (0.07)	0.66^{b} (0.09)
C20:1 c9	1.92^{a} (0.37)	2.35^{a} (0.44)	3.94^{b} (0.60)	4.37^{b} (0.80)
C20:1 c11	2.79^{a} (0.79)	3.43^{a} (0.63)	1.21^{b} (0.20)	1.41^{b} (0.30)
C22:1 c9	0.10 (0.03)	0.14 (0.02)	0.10 (0.02)	0.11 (0.02)
C24:1	0.08 (0.01)	0.10 (0.03)	0.07 (0.01)	0.09 (0.05)
PUFA	275.73^{a,b} (49.14)	324.67^{b} (54.96)	255.31^{b} (38.89)	261.10^{b} (34.10)
C16:2 c9t12	0.02 (0.01)	0.03 (0.01)	0.02 (0.01)	0.02 (0.01)
C16:2 c9c12	0.07^{a} (0.01)	0.08^{a} (0.03)	0.05^{b} (0.01)	0.04^{b} (0.01)
C18:2 t9t12	0.21 (0.04)	0.24 (0.04)	0.22 (0.03)	0.25 (0.03)
C18:2 c9t12	0.07^{a} (0.02)	0.08^{a} (0.03)	0.13^{b} (0.03)	0.15^{b} (0.02)
C18:2 t9c12	0.68^{a} (0.11)	0.72^{a} (0.12)	0.41^{b} (0.08)	0.43^{b} (0.06)
C18:2 c9c12	253.66^{a,b} (44.95)	299.16^{b} (51.39)	230.87^{a} (35.64)	236.42^{a} (31.56)
C18:2 c9c15	n.d.^{a}	n.d.^{a}	0.26^{b} (0.07)	0.27^{b} (0.04)
C18:3 t9t12c15	0.36 (0.06)	0.40 (0.06)	0.44 (0.06)	0.49 (0.07)
C18:3 c6c9c12	0.96 (0.15)	1.02 (0.19)	0.93 (0.14)	1.04 (0.13)
C18:3 c9t12t15	0.31^{a} (0.06)	0.43^{a} (0.08)	0.51^{b} (0.07)	0.55^{b} (0.09)
C18:3 c9c12c15	9.55^{a,b} (2.16)	11.22^{b} (2.20)	8.61^{a} (1.55)	8.53^{a} (1.32)
C18:2 c9t11	0.29^{a} (0.13)	0.28^{a} (0.10)	0.89^{b} (0.15)	0.96^{b} (0.14)
C20:2 c11c14	1.58^{a} (0.40)	1.81^{a} (0.18)	4.38^{b} (0.58)	4.42^{b} (0.68)
C20:3 c8c11c14	0.82^{a} (0.22)	0.93^{a,b} (0.14)	1.09^{b,c} (0.16)	1.20^{c} (0.20)
C20:4 AA	4.79 (0.98)	5.46 (1.06)	4.24 (0.80)	4.16 (0.55)
C20:3 c11c14c17	0.16^{a} (0.04)	0.19^{a} (0.06)	0.46^{b} (0.07)	0.44^{b} (0.06
C20:5 n3	0.21^{a,b} (0.06)	0.24^{b} (0.08)	0.17^{a} (0.04)	0.16^{a} (0.03)
C22:2 c13c16	0.12^{a,b} (0.02)	0.14^{b} (0.05)	0.10^{a} (0.02)	0.11^{a} (0.04)
C22:5 n6	0.53^{a} (0.12)	0.64^{a} (0.16)	0.33^{b} (0.06)	0.36^{b} (0.07)
C22:5 n3	0.61 (0.21)	0.76 (0.27)	0.62 (0.16)	0.56 (0.12)
C22:6 DHA	0.73^{a} (0.21)	0.82^{a} (0.19)	0.58^{b} (0.13)	0.56^{b} (0.12)
μg mg^{-1}	738.25^{a} (101.11)	854.86^{a} (102.26)	926.67^{b} (137.22)	996.42^{b} (133.87)

c: *cis* double bond; t: *trans* double bond; AA: arachidonic acid; n3: omega 3 fatty acid; n6: omega 6 fatty acid; DHA: docosahexanoic fatty acid. Standard diet (C); standard diet + blackberry anthocyanin extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanin extract (HFBE). Values are expressed as mean (SD). ^{a,b,c,d} Superscript letters in a row for significant differences among groups ($p < 0.05$) after one-way ANOVA followed by Bonferroni adjustment.

diet and BE supplementation decreased BDNF levels, but the mechanisms behind this same effect may be different.

In previous animal studies supplementation with blueberries, also rich in anthocyanins, or with the flavonoids alone, for six weeks resulted in increased hippocampal levels of BDNF.[50,51] Supplementation with BE, although decreasing BDNF levels, showed increased dopamine turnover in the striatum. Previous studies have suggested an association between glucose and dopamine levels,[52] with low levels of glucose in the brain inhibiting dopamine release.[52,53] Interference of flavonoids with glucose transporters, which has already been shown in previous reports[31,54] and includes modulation of glucose access to the brain, could play a role in the dopamine changes observed in this study.

The leptin/adiponectin ratio has been correlated with metabolic parameters and predictor of cardiovascular risk.[55] Diet increased this ratio with no effects of BE supplementation. These animals have also increased production of adiponectin and leptin, produced by adipose tissue which is known to be involved in metabolic regulation.

Anthocyanins have been suggested as potentially increasing plasma long chain fatty acid levels, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the main very long-chain (n-3) PUFA,[56] although this issue remains controversial.[57] In the present study, animals fed with the anthocyanin-rich extract did not show any difference in long-chain fatty acid composition. Although the biological significance was not clear, BE significantly increased methylhexadecanoic acid

Table 9 Fatty acid composition (μg FA per mg tissue) in liver samples

	C	BE	HF	HFBE
SFA	11.05 (1.54)	10.47 (1.14)	12.87 (2.54)	12.30 (2.34)
C14	0.09^{a} (0.02)	0.09^{a} (0.02)	0.13^{b} (0.06)	0.14^{b} (0.04)
C15	0.05 (0.00)	0.05 (0.01)	0.05 (0.01)	0.04 (0.01)
C16	5.70 (0.82)	5.45 (0.71)	6.46 (1.54)	6.10 (1.49)
C17	0.11 (0.01)	0.11 (0.02)	0.11 (0.02)	0.10 (0.02)
C18	$4.91^{a,b}$ (0.75)	4.58^{b} (0.59)	5.90^{a} (1.25)	5.70^{a} (0.95)
C24	0.14 (0.03)	0.14 (0.03)	0.19 (0.05)	0.18 (0.06)
MUFA	3.47 (0.64)	3.36 (0.76)	6.79 (2.61)	6.60 (2.01)
C81:1 t	0.05 (0.01)	0.04 (0.01)	0.10 (0.03)	0.10 (0.03)
C16:1 c7	0.05^{a} (0.01)	0.05^{a} (0.01)	0.12^{b} (0.06)	0.12^{b} (0.06)
C16:1 c9	0.44^{a} (0.04)	0.49^{a} (0.10)	0.29^{b} (0.08)	$0.36^{a,b}$ (0.14)
C18:1 c9	1.75^{a} (0.50)	1.53^{a} (0.49)	5.42^{b} (2.23)	5.17^{b} (1.62)
C18:1 c11	1.08^{a} (0.13)	1.16^{a} (0.17)	0.75^{b} (0.21)	0.74^{b} (0.19)
C18:1 t16	0.05^{a} (0.01)	0.05^{a} (0.01)	0.03^{b} (0.01)	0.03^{b} (0.00)
C20:1 c9	0.03^{a} (0.02)	0.04^{a} (0.01)	0.09^{b} (0.03)	0.08^{b} (0.03)
C20:1 c11	0.07^{a} (0.01)	0.06^{a} (0.01)	0.03^{b} (0.01)	0.03^{b} (0.01)
PUFA	11.63 (1.51)	11.26 (1.54)	12.97 (2.67)	12.24 (1.91)
C18:2 c9c12	4.42 (0.44)	4.15 (0.51)	5.70 (1.48)	5.06 (1.14)
C18:3 c6c9c12	$0.08^{a,b}$ (0.02)	0.06^{b} (0.01)	0.11^{a} (0.03)	0.11^{a} (0.03)
C18:3 c9c12c15	0.07^{a} (0.02)	0.05^{a} (0.02)	0.13^{b} (0.05)	0.12^{b} (0.03)
C20:2 c11c14	0.09^{a} (0.01)	0.10^{a} (0.02)	0.14^{b} (0.03)	0.14^{b} (0.03)
C20:3 c8c11c14	0.20 (0.03)	0.22 (0.04)	0.19 (0.04)	0.21 (0.04)
C20:4 AA	5.64 (1.19)	5.54 (1.14)	5.35 (1.50)	5.28 (1.11)
C20:3 c11c14c17	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)
C20:5 n3	0.05 (0.01)	0.05 (0.01)	0.04 (0.01)	0.04 (0.01)
C22:5 n6	0.11 (0.02)	0.11 (0.04)	0.10 (0.02)	0.12 (0.02)
C22:5 n3	0.17 (0.03)	0.19 (0.04)	0.17 (0.03)	0.16 (0.04)
C22:6 DHA	0.75^{a} (0.12)	0.74^{a} (0.17)	1.00^{b} (0.25)	0.97^{b} (0.18)
μg mg^{-1}	$26.15^{a,b}$ (2.97)	25.09^{b} (2.57)	32.63^{a} (6.91)	31.14^{a} (5.78)

c: *cis* double bond; t: *trans* double bond; AA: arachidonic acid; n3: omega 3 fatty acid; n6: omega 6 fatty acid; DHA: docosahexanoic fatty acid. Standard diet (C); standard diet + blackberry anthocyanin extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanin extract (HFBE). Values are expressed as mean (SD). ^{a,b,c,d} Superscript letters in a row for significant differences among groups ($p < 0.05$) after one-way ANOVA followed by Bonferroni adjustment.

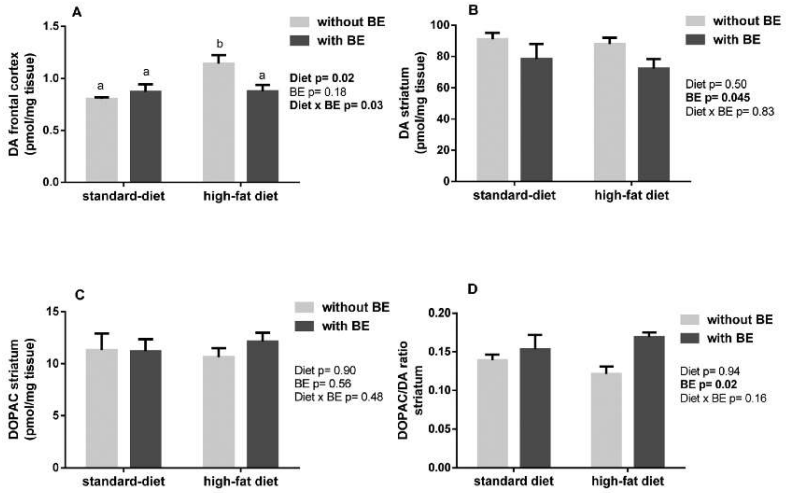

Fig. 6 Effects of blackberry anthocyanin extract (BE) on the release of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) on Wistar rats fed a standard or high-fat diet for 17 weeks ($n = 6$). Results are expressed as mean ± SEM. Statistical significance was tested by two-way ANOVA considering diet and blackberry supplementation as factors, followed by Fisher's LSD test in the prefrontal cortex. Statistical significance was considered when $p < 0.05$.

(C17i) in standard fed animals in both adipose tissue deposits. Branched-chain FAs are ubiquitous in nature and present in particularly large quantities in bacteria, but are rarely found in other organisms.[58] This fact, coupled with a lack of detection of this fatty acid on the standard diet (data not shown), suggested that somehow BE could be changing either the animals' metabolism or the production of fatty acids by intestinal bacteria.[59]

The effect of flavonoids, similar to that of many other xenobiotics, varies with the supplemented dose, many times without a dose-dependency, especially regarding *in vivo* effects.[60,61] The dose given to the animals – 25 mg/kg body weight – would correspond to approximately 243 mg of blackberry extract in a human adult weighing 60 kg, using the formula to human equivalent dose (HED) based on body surface area as described by Reagan-Shaw.[62] This dose could be easily achieved by diet, by eating as much as 100 g of blackberries a day,[63] or could also be introduced as a food supplement if its risk–benefit so justifies. Future studies should test different doses to maximize the potential of blackberry supplementation whilst minimizing undesirable effects.

This study confirmed that high-fat high-carbohydrate diet-induced obesity can prompt several features of metabolic dysfunction in Wistar rats, some of them being partially reverted with low doses of blackberry extract supplementation for a long period. A decrease of plasma lactate levels appeared to be the strongest effect of blackberry supplementation, independent of the fat content of the diet. Blackberry supplementation was also able to modulate levels of dopamine and its clearance while reducing BDNF levels, the biological relevance of which is still a matter of debate. Further interventional studies should clarify these outcomes. The interest in effective intervention strategies to prevent/treat obesity and related pathologies has been increasing, along with a recent interest regarding the close associations between obesity and brain dysfunction. These results have advanced the knowledge of the therapeutic potential of berries, and may empower the achievement of specific recommendations for berry intake or purified blackberry extract in the future.

Authors' contributions

MM contributed to the experimental design, data acquisition and analysis and drafted the manuscript. CM, SN, JF and LA contributed to data acquisition. IF and NM were responsible for the preparation of the blackberry extracts. AF and CC were responsible for study conception, conduction of experiments, data interpretation, preparation and critical revision of the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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References

1 A. Berghofer, T. Pischon, T. Reinhold, C. M. Apovian, A. M. Sharma and S. N. Willich, *BMC Public Health*, 2008, **8**, 200.
2 C. L. Ogden, M. D. Carroll, B. K. Kit and K. M. Flegal, *JAMA, J. Am. Med. Assoc.*, 2014, **311**, 806–814.
3 A. K. Dahl and L. B. Hassing, *Epidemiol. Rev.*, 2012, DOI: 10.1093/epirev/mxs002.
4 F. Muller-Riemenschneider, T. Reinhold, A. Berghofer and S. N. Willich, *Eur. J. Epidemiol.*, 2008, **23**, 499–509.
5 G. A. Bray, *J. Clin. Endocrinol. Metab.*, 2004, **89**, 2583–2589.
6 M. A. Beydoun, H. A. Beydoun and Y. Wang, *Obes. Rev.*, 2008, **9**, 204–218.
7 M. Prince, R. Bryce, E. Albanese, A. Wimo, W. Ribeiro and C. P. Ferri, *Alzheimer's Dementia*, 2013, **9**, 63–75.
8 S. E. Kanoski and T. L. Davidson, *Physiol. Behav.*, 2011, **103**, 59–68.
9 H. R. Park, M. Park, J. Choi, K. Y. Park, H. Y. Chung and J. Lee, *Neurosci. Lett.*, 2010, **482**, 235–239.
10 J. K. Morris, G. L. Bomhoff, B. K. Gorres, V. A. Davis, J. Kim, P. P. Lee, W. M. Brooks, G. A. Gerhardt, P. C. Geiger and J. A. Stanford, *Exp. Neurol.*, 2011, **231**, 171–180.
11 B. Buijsse, E. J. Feskens, M. B. Schulze, N. G. Forouhi, N. J. Wareham, S. Sharp, D. Palli, G. Tognon, J. Halkjaer, A. Tjonneland, M. U. Jakobsen, K. Overvad, A. D. van der, H. Du, T. I. Sorensen and H. Boeing, *Am. J. Clin. Nutr.*, 2009, **90**, 202–209.
12 M. González-Castejón and A. Rodriguez-Casado, *Pharmacol. Res.*, 2011, **64**, 438–455.
13 L. Letenneur, C. Proust-Lima, A. Le Gouge, J. F. Dartigues and P. Barberger-Gateau, *Am. J. Epidemiol.*, 2007, **165**, 1364–1371.
14 X. Gao, A. Cassidy, M. Schwarzschild, E. Rimm and A. Ascherio, *Neurology*, 2012, **78**, 1138–1145.
15 S. Norberto, S. Silva, M. Meireles, A. Faria, M. Pintado and C. Calhau, *J. Funct. Foods*, 2013, **5**, 1518–1528.
16 A. Basu, M. Rhone and T. J. Lyons, *Nutr. Rev.*, 2010, **68**, 168–177.
17 C. Rendeiro, J. D. Guerreiro, C. M. Williams and J. P. Spencer, *Proc. Nutr. Soc.*, 2012, **71**, 246–262.

18 B. O. Cho, H. W. Ryu, C. H. Jin, D. S. Choi, S. Y. Kang, D. S. Kim, M. W. Byun and I. Y. Jeong, *J. Agric. Food Chem.*, 2011, **59**, 11442–11448.

19 B. Shukitt-Hale, V. Cheng and J. A. Joseph, *Nutr. Neurosci.*, 2009, **12**, 135–140.

20 N. M. Hassimotto and F. M. Lajolo, *J. Sci. Food Agric.*, 2011, **91**, 523–531.

21 I. Serraino, L. Dugo, P. Dugo, L. Mondello, E. Mazzon, G. Dugo, A. P. Caputi and S. Cuzzocrea, *Life Sci.*, 2003, **73**, 1097–1114.

22 J. Azevedo, I. Fernandes, A. Faria, J. Oliveira, A. Fernandes, V. de Freitas and N. Mateus, *Food Chem.*, 2010, **119**, 518–523.

23 J. Oliveira, V. Fernandes, C. Miranda, C. Santos-Buelga, A. Silva, V. de Freitas and N. Mateus, *J. Agric. Food Chem.*, 2006, **54**, 6894–6903.

24 I. Fernandes, J. Azevedo, A. Faria, C. Calhau, V. de Freitas and N. Mateus, *J. Agric. Food Chem.*, 2009, **57**, 735–745.

25 C. Barrientos, R. Racotta and L. Quevedo, *Nutr. Res.*, 2010, **30**, 791–800.

26 C. van den Brom, C. Bulte, B. Kloeze, S. Loer, C. Boer and R. Bouwman, *Cardiovasc. Diabetol.*, 2012, **11**, 74.

27 J. Ibrahim, B. C. Berk and A. D. Hughes, *Clini. Exp. Hypertens.*, 2006, **28**, 57–72.

28 K. Rutter, L. Hennoste, L. C. Ward, B. H. Cornish and B. J. Thomas, *Lab. Anim.*, 1998, **32**, 65–71.

29 P. Castro-Gómez, J. Fontecha and L. M. Rodríguez-Alcalá, *Talanta*, 2014, **128**, 518–523.

30 P. Soares-Da-Silva and M. H. Fernandes, *Acta Physiol. Scand.*, 1991, **143**, 287–293.

31 A. Faria, D. Pestana, J. Azevedo, F. Martel, V. de Freitas, I. Azevedo, N. Mateus and C. Calhau, *Mol. Nutr. Food Res.*, 2009, **53**, 1430–1437.

32 P. V. A. Babu, D. Liu and E. R. Gilbert, *J. Nutr. Biochem.*, 2013, **24**, DOI: 10.1016/j.jnutbio.2013.1006.1003.

33 M. Itoh, T. Suganami, R. Hachiya and Y. Ogawa, *Int. J. Inflammation*, 2011, **2011**, 720926.

34 I. J. Neeland, C. R. Ayers, A. K. Rohatgi, A. T. Turer, J. D. Berry, S. R. Das, G. L. Vega, A. Khera, D. K. McGuire, S. M. Grundy and J. A. de Lemos, *Obesity*, 2013, **21**, 19.

35 S. K. Panchal, L. Ward and L. Brown, *Eur. J. Nutr.*, 2013, **52**, 559–568.

36 X.-Y. Li, Z.-X. Zhao, M. Huang, R. Feng, C.-Y. He, C. Ma, S.-H. Luo, J. Fu, B.-Y. Wen, L. Ren, J.-W. Shou, F. Guo, Y. Chen, X. Gao, Y. Wang and J.-D. Jiang, *J. Transl. Med.*, 2015, **13**, 278.

37 J. Lovejoy, F. D. Newby, S. S. Gebhart and M. DiGirolamo, *Metabolism*, 1992, **41**, 22–27.

38 M. DiGirolamo, F. D. Newby and J. Lovejoy, *FASEB J.*, 1992, **6**, 2405–2412.

39 G. van Hall, *Acta Physiol.*, 2010, **199**, 499–508.

40 K. Matsushita, E. K. Williams, M. L. Mongraw-Chaffin, J. Coresh, M. I. Schmidt, F. L. Brancati, R. C. Hoogeveen, C. M. Ballantyne and J. H. Young, *Am. J. Epidemiol.*, 2013, **178**, 401–409.

41 A. B. Klein, R. Williamson, M. A. Santini, C. Clemmensen, A. Ettrup, M. Rios, G. M. Knudsen and S. Aznar, *Blood BDNF concentrations reflect brain-tissue BDNF levels across species*, 2011.

42 K. Miyazaki, N. Narita, R. Sakuta, T. Miyahara, H. Naruse, N. Okado and M. Narita, *Brain Dev.*, 2004, **26**, 292–295.

43 S.-H. Shim, Y. Hwangbo, Y.-J. Kwon, H.-Y. Jeong, B.-H. Lee, H.-J. Lee and Y.-K. Kim, *Prog. Neuro-Psychopharmacol. Biol. Psychiatry*, 2008, **32**, 1824–1828.

44 A. H. El-Gharbawy, D. C. Adler-Wailes, M. C. Mirch, K. R. Theim, L. Ranzenhofer, M. Tanofsky-Kraff and J. A. Yanovski, *J. Clin. Endocrinol. Metab.*, 2006, **91**, 3548–3552.

45 E. Gajewska, M. Sobieska, D. Lojko, K. Wieczorowska-Tobis and A. Suwalska, *Eur. Rev. Med. Pharmacol. Sci.*, 2014, **18**, 3246–3250.

46 K. Sakata and S. M. Duke, *Neuroscience*, 2014, **260**, 265–275.

47 J. A. Siuciak, C. Boylan, M. Fritsche, C. A. Altar and R. M. Lindsay, *Brain Res.*, 1996, **710**, 11–20.

48 C. A. Altar, C. B. Boylan, C. Jackson, S. Hershenson, J. Miller, S. J. Wiegand, R. M. Lindsay and C. Hyman, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 11347–11351.

49 F. Fumagalli, G. Racagni, E. Colombo and M. A. Riva, *Mol. Psychiatry*, 2003, **8**, 898–899.

50 C. Rendeiro, D. Vauzour, M. Rattray, P. Waffo-Teguo, J. M. Merillon, L. T. Butler, C. M. Williams and J. P. Spencer, *PLoS One*, 2013, **8**, e63535.

51 C. Rendeiro, D. Vauzour, R. J. Kean, L. T. Butler, M. Rattray, J. P. Spencer and C. M. Williams, *Psychopharmacology*, 2012, **223**, 319–330.

52 K. Blum, P. K. Thanos and M. S. Gold, *Front. Psychol.*, 2014, **5**, 919.

53 C. M. Adler, I. Elman, N. Weisenfeld, L. Kestler, D. Pickar and A. Breier, *Neuropsychopharmacology*, 2000, **22**, 545–550.

54 M. Meireles, F. Martel, J. Araujo, C. Santos-Buelga, S. Gonzalez-Manzano, M. Duenas, V. de Freitas, N. Mateus, C. Calhau and A. Faria, *J. Membr. Biol.*, 2013, **246**, 669–677.

55 G. D. Norata, S. Raselli, L. Grigore, K. Garlaschelli, E. Dozio, P. Magni and A. L. Catapano, *Stroke*, 2007, **38**, 2844–2846.

56 M. C. Toufektsian, P. Salen, F. Laporte, C. Tonelli and M. de Lorgeril, *J. Nutr.*, 2011, **141**, 37–41.

57 D. Vauzour, N. Tejera-Hernandez, C. O'Neill, V. Booz, B. Jude, I. Ernst, N. Rigby, J. M. Silvan, P. Curtis, A. Cassidy, S. de Pascual-Teresa, G. Rimbach and A. M. Minihane, *J. Nutr. Biochem.*, DOI: 10.1016/j.jnutbio.2014.09.005.

58 M. Kniazeva, Q. T. Crawford, M. Seiber, C. Y. Wang and M. Han, *PLoS Biol.*, 2004, **2**, E257.

59 A. Faria, I. Fernandes, S. Norberto, N. Mateus and C. Calhau, *J. Agric. Food Chem.*, 2014, **62**, 6898–6902.

60 E. J. Calabrese and R. B. Blain, *Regul. Toxicol. Pharmacol.*, 2011, **61**, 73–81.

61 A. Murakami, *Arch. Biochem. Biophys.*, 2014, **557**, 3–10.

62 S. Reagan-Shaw, M. Nihal and N. Ahmad, *FASEB J.*, 2008, **22**, 659–661.

63 X. Wu, G. R. Beecher, J. M. Holden, D. B. Haytowitz, S. E. Gebhardt and R. L. Prior, *J. Agric. Food Chem.*, 2006, **54**, 4069–4075.

CHAPTER V

"The impact of chronic blackberry intake on neuroinflammatory status of rats fed a standard or high-fat diet"

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The impact of chronic blackberry intake on the neuroinflammatory status of rats fed a standard or high-fat diet

Manuela Meireles[a,b], Cláudia Marques[a], Sónia Norberto[a], Iva Fernandes[c], Nuno Mateus[c], Catarina Rendeiro[b], Jeremy P.E. Spencer[b], Ana Faria[a,c,d], Conceição Calhau[a,e,*]

[a]Departamento de Bioquímica, Faculdade de Medicina, Universidade do Porto, 4200-319 Porto, Portugal
[b]Department of Food Biosciences, School of Chemistry, Food and Pharmacy, University of Reading, Reading RG6 6AP, UK
[c]REQUIMTE — Rede de Química e Tecnologia, Faculdade de Ciências, Universidade do Porto, 4169-009 Porto, Portugal
[d]Faculdade Ciências da Nutrição e Alimentação, Universidade do Porto, 4200-465 Porto, Portugal
[e]CINTESIS — Center for Research in Health Technologies and Information Systems, Centro de Investigação Médica, Faculdade de Medicina, Universidade do Porto, 4200-319 Porto, Portugal

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Abstract

Neuroinflammation has been suggested as a central mediator of central nervous system dysfunction, including in dementia and neurodegenerative disease. Flavonoids have emerged as promising candidates for the prevention of neurodegenerative diseases and are thought to be capable of antiinflammatory effects in the brain. In the present study, the impact of a chronic intake of an anthocyanin extract from blackberry (BE) on brain inflammatory status in the presence or absence of a high-fat diet was investigated. Following intake of the dietary regimes for 17 weeks neuroinflammatory status in Wistar rat cortex, hippocampus and plasma were assessed using cytokine antibody arrays. In the cortex, intake of the high-fat diet resulted in an increase of at least 4-fold, in expression of the cytokine-induced neutrophil chemoattractant CINC-3, the ciliary neurotrophic factor CNTF, the platelet-derived growth factor PDGF-AA, IL-10, the tissue inhibitor of metalloproteinase TIMP-1 and the receptor for advanced glycation end products RAGE. BE intake partially decreased the expression of these mediators in the high-fat challenged brain. In standard-fed animals, BE intake significantly increased cortical levels of fractalkine, PDGF-AA, activin, the vascular endothelial growth factor VEGF and agrin expression, suggesting effects as neuronal growth and synaptic connection modulators. In hippocampus, BE modulates fractalkine and the thymus chemokine TCK-1 expression independently of diet intake and, only in standard diet, increased PDGF-AA. Exploring effects of anthocyanins on fractalkine transcription using the neuronal cell line SH-SY5Y suggested that other cell types may be involved in this effect. This is the first evidence, in *in vivo* model, that blackberry extract intake may be capable of preventing the detrimental effects of neuroinflammation in a high-fat challenged brain. Also, fractalkine and TCK-1 expression may be specific targets of anthocyanins and their metabolites on neuroinflammation.

Keywords: Anthocyanins; Cytokines; Flavonoids; Fractalkine; High-fat diet; Neuroinflammation

1. Introduction

The incidence of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) and dementia, have become more prevalent due, in part, to the exponential increase in life expectancy, making the discovery of novel treatments most pressing [1]. Both typical and atypical brain aging are associated with an increase in neuroinflammation and may contribute to the dysfunction of the central nervous system (CNS) and lead to the early onset of dementia and/or neurodegenerative diseases [2,3]. In support of this, the use of nonsteroidal antiinflammatory drugs has been shown to partly delay or prevent the onset of some neurodegenerative diseases [4,5]. On the other hand, modifiable lifestyle factors, such as diet, have also been postulated to have the potential to protect or stimulate neuroinflammation [6,7]. Notably, high-fat diets, combined with physical inactivity, may lead to obesity [6], a major risk factor for metabolic and cognitive dysfunction [8–10]. For example, intake of a typical 'western' diet (high fat; high sugar) has been shown to compromise normal cognitive functioning [10,11]. Furthermore, high-fat diets have been shown to induce markers of brain inflammation, with proinflammatory actions in the cerebral cortex (prostaglandin E2 levels and ciclooxigenases 1 and 2 expression) and hippocampus (tumor necrosis factor TNF-α, interleukins IL-6 and IL-1β and macrophage chemoattractant protein MCP-1) [12–14].

A growing body of evidence suggests that flavonoid intake may attenuate the progression of neurodegenerative disorders [15,16]. In particular, prospective studies showed a positive association between flavonoid intake and reduction in cognitive decline with age [17] and decrease risk of AD [18]. Furthermore, the higher intake of flavonoids (flavanones, anthocyanins, flavan-3-ols, flavonols, flavones) has been shown to be associated with a reduced risk of developing PD, in

* Corresponding author at: Department of Biochemistry, Faculty of Medicine of the University of Porto, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal. Tel./fax: +351-22-551-36-24.
E-mail address: ccalhau@med.up.pt (C. Calhau).

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particular, with anthocyanins and the intake of anthocyanin-rich foods such as berries [19]. Although the molecular mechanisms underlying such protective effects remain unclear, the antiinflammatory properties of flavonoids are likely to play an important role in driving these positive outcomes [20–23]. Indeed, flavonoids and their *in vivo* metabolites have been shown to protect neurons against neuroinflammatory injury [20,24].

Despite the growing body of studies investigating the impact of flavonoids in brain function, only a few animal investigations have focussed on their specific actions in the context of neuroinflammation [25–28]. Anthocyanins from berries have aroused particular interest as potential neuroprotectants [29,30]. As such, the present study was designed to investigate the impact of long-term blackberry anthocyanin intake on neuroinflammation status of both high-fat-fed and low-fat-fed rats. A simultaneous multiple cytokine detection approach was used first in two important regions of CNS, cortex and hippocampus, and extended to plasma to explore relation with systemic cytokine profile. From the results of cytokine arrays, further hypotheses were explored, namely, the interaction of blackberry extract with microglia phenotype activation and the modulation of the cytokine fractalkine in an *in vitro* model of neurons.

2. Methods

2.1. Blackberry extract and anthocyanin metabolite synthesis

Preparation of anthocyanin blackberry extract was achieved using previously described methods [31,32]. Briefly, blackberries (*Rubus fruticosus*) were extracted with 50% aqueous ethanol (pH 1.5, acidified with HCl) for 24 h at 22°C. The solution obtained was filtered (50-μm nylon membrane) and concentrated using a rotary evaporator under at 30°C. The concentrated extract was added to a polyamide gel column (mesh 100–120) to remove sugars. The sugar-free anthocyanin extract was freeze-dried and stored at −20°C. The anthocyanin extract was analyzed by HPLC at 520 nm, as previously described [33]. Following preparative HPLC, the purified extract was characterized for anthocyanin content by analytical HPLC coupled to UV–vis, DAD-ESI/MS and NMR techniques. The mixture 3′ and 4′-Me-Cy3glc (Peo3glc and IsoPeo3glc) was obtained by enzymatic hemisynthesis. Briefly, Cy3glc was incubated with rat liver cytosol protein fraction and *S*-adenosyl-L-methionine during 2 h at 37°C. The identity of the purified compounds present in the mixture was established by UV–vis, HPLC-DAD-ESI/MS and NMR techniques as previously described [34].

2.2. Study design

Twenty-four male Wistar rats (200–250 g body weight) were acquired from Harlan Laboratories (Santiga, Spain). Animals were maintained at 23–25°C on a 12/12 h light–dark cycle and housed two per cage. Animal handling and housing protocols followed European Union guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals. The study was approved by Ethical Committee of Faculty of Medicine University of Porto and São João Hospital Center. After 2 weeks of acclimatization, animals were divided into four groups (n=6 rats per group): standard diet (C); standard diet+blackberry anthocyanin extract (BE); high-fat diet (HF); and high-fat diet+blackberry anthocyanin extract (HFBE). Animals were fed *ad libitum* with a standard maintenance diet (4% fat) from Harlan (2014S, Teklad Diets; Harlan Laboratories) or a high-fat chow diet (45% fat) from Research Diet D12451 (New Brunswick, NJ, USA) for 17 weeks. The blackberry anthocyanin dose (25 mg kg^{-1} body weight; adjusted weekly for weight changes) was incorporated into the animal feeds and was changed daily. Animal body weight and food ingestion were measured twice a week. Following the 17-week intervention, animals were anesthetized with ketamine+xylazine (50 mg kg^{-1}+1 mg kg^{-1}) and kept under isoflurane during blood collection from left ventricle. Blood was collected with heparinized needles and the protease inhibitor PMSF (100 μM) was added. After blood centrifugation (2000*g*; 15 min), plasma was collected and stored at −20°C. Rats were decapitated for brain removal. Hippocampus and cortex were immediately frozen in liquid nitrogen and kept at −80°C until use.

2.3. Cytokine antibody arrays

In order to have a global view of cytokines expression in brain, predefined cytokine arrays were used in cortex (RayBio Rat Cytokine Antibody Array 2; RayBiotech, Norcross, GA, USA) and plasma (Rat Cytokine Array Panel A; R&D Systems Europe, UK). Thirty microliters of plasma from each animal treatment was mixed, diluted and used according to instructions. For the brain cortex array, cortex tissue from animals in each experimental group was mixed, proteins were extracted according to kit and was quantified with Bradford reagent (Bio-Rad Laboratories, Hertfordshire, UK) and a 200-μg protein were used. Chemiluminescent detection was achieved using a luminescent image analyzer (ImageQuant LAS 4000mini from GE Healthcare, Life Sciences) and data were analyzed with ImageQuant TL array software, version 8.1.

Protein extraction from hippocampus was made with a Mammalian Tissue Lysis, CelLyticMT (C-3228; Sigma-Aldrich, Dorset, UK). A ratio of 1:20 was used (1 mg tissue/20 μl reagent), and PMSF (phenylmethylsulfonyl fluoride; Sigma-Aldrich, Dorset, UK), a protease inhibitor, was added to the reagent before use, at a final concentration of 100 μM. After a centrifugation of 15,000*g* for 10 min, supernatant was collected and proteins were measured with Bradford reagent (Bio-Rad Laboratories), according to previous literature [29]. For the determinations in the hippocampus, a glass-based array (Quantibody Rat Cytokine Array; RayBiotech) was performed according to manufacturer's instructions. Arrays were incubated with the samples (250 μg ml^{-1}) or standards and cytokine concentration in samples was determined using a standard curve. Fluorescent detection was analyzed with laser scanner (Genepix Professional 4200A; Molecular Devices) and data were extracted with GenePix Pro 6 Microarray Acquisition & Analysis Software (from Molecular Devices).

2.4. Measurements of microglia activation markers in cortex

In order to characterize microglia polarization, expression of IL-1β (marker of M1 status) and arginase activity (marker of M2 activation) were explored [35]. Cortex tissue was lysed and homogenized with Mammalian Tissue Lysis, CelLyticMT (C-3228; Sigma-Aldrich, Dorset, UK) in a ratio of 1 mg tissue:20 μl lysis buffer. The protease inhibitor PMSF (100 μM) was added, homogenates were centrifuged at 15,000*g* for 10 min and the supernatant was retained. Protein content of the supernatants was assessed by the Bradford assay (Bio-Rad Laboratories). Arginase activity was measured using a commercial arginase assay kit (KA1609; Abnova, Taipei City, Taiwan). Standards, controls and 5-fold diluted samples were incubated with arginase substrate for 2 h at 37°C then reaction was stopped with urea reagent and kept for 60 min before optical measurement at 450 nm.

Levels of IL-1β were measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (RayBio Rat IL-1; tebu-bio, Portugal). Cortex supernatant 4-fold diluted, standards and controls were plated in duplicate on a 96-well microplate coated with anti-rat IL-1β polyclonal antibody and incubated for overnight at 4°C. Biotinylated anti-IL-1β monoclonal antibody was then added for 1 h. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was added for 45 min. The wells were again washed, and a TMB substrate solution was added allowing color to develop in proportion to the amount of IL-1β

Table 1
Effect of 17 weeks of consumption of blackberry anthocyanin extract (25 mg kg^{-1} body weight) on body weight, food and energy intake.

	C	BE	HF	HFBE
Initial body weight	237.8±7.7	246.5±8.6	237.2±7.2	244.0±11.1
Final body weight	429.8±27.8[a]	432.7±28.4[a]	550.7±67.6[b]	581.0±63.1[b]
Food intake (g/day)	18.2±1.2[a]	17.8±2.1[a]	15.7±1.1[b]	17.4±1.4[a]
Energy intake (kcal/day)	52.8±3.5[a]	51.7±6.2[a]	74.4±3.5[b]	82.1±6.7[c]

(C) Standard diet; (BE) standard diet+blackberry anthocyanins extract; (HF) high-fat diet or (HFBE) high-fat diet+blackberry anthocyanins extract group. Values are expressed as mean±SD. Superscript letters represent statistically significant differences ($P<.05$).

bound. Stop solution was added and changed the color from blue to yellow. Intensity of the color was measured by optical density at 450 nm and calculated according to standard curve.

2.5. Cell line and culture conditions

The SH-SY5Y cell line is commonly used as a neuronal *in vitro* model [36]. The cell line was obtained from the American Type Culture Collection (CRL-2266; Rockville, MD, USA) and was used between passage number 11 and 12. Cells were maintained in a humidified atmosphere of 5% CO_2/95% air and were grown in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich, Madrid, Spain), supplemented with 1.19 g l^{-1} $NaHCO_3$, 3.47 g l^{-1} Hepes, 5% fetal bovine serum, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B (all from Sigma-Aldrich). Cells were cultured with vehicle (DMSO, 0.01%) or the compounds cyanidin-3-glucoside and cyanidin (both from Extrasynthèse, Genay, France) or a mixture of two of its phase I/II metabolites (3′-Me-Cy3glc+4′-Me-Cy3glc) or phenolic acids (Sigma-Aldrich, Madrid, Spain) for 24 h in three independent experiments.

Table 2
Effect of 17 weeks of consumption of high-fat diet (45% fat) by Wistar rats on cytokines expression on brain cortex.

Protein	HF expression vs. C %	Rank product
PDGF-AA	**538.5±41.7**	**0.002**
CNTF	**664.1±185.1**	**0.002**
CINC-3	**492.6±30.8**	**0.010**
IL-10	**460.1±6.1**	**0.017**
TIMP-1	**432.1±92.4**	**0.021**
RAGE	**417.9±11.7**	**0.030**
CINC-1	385.1±67.6	0.052
IL-1 R6	366.2±0.1	0.054
Prolactin R	350.2±27.2	0.062
TCK-1	320.3±11.4	0.095
VEGF	283.8±9.5	0.145
IL-1β	268.8±102.3	0.151
IL-4	270.5±21.3	0.180
CINC-2α	281.3±29.1	0.181
Activin A	279.5±25.6	0.187
L-Selectin	266.7±28.9	0.236
ICAM-1	250.8±11.4	0.260
IL-6	256.7±33.5	0.266
IFN-γ	248.3±13.2	0.272
LIX	246.6±9.7	0.277
MMP-8	233.2±7.0	0.343
IL-2	233.1±22.9	0.353
Fractalkine	221.3±26.9	0.368
MIP-3α	215.4±21.8	0.427
CD86	207.7±0.1	0.497
IL-13	190.4±6.7	0.561
Fas ligand	186.0±27.6	0.577
GM-CSF	169.3±6.3	0.654
Leptin	143.3±41.2	0.765
TNF-α	148.3±10.9	0.775
IL-1α	149.4±7.3	0.804
MCP-1	140.6±19.4	0.830
Agrin	144.8±7.8	0.885
β-NGF	126.8±8.5	0.971

(C) Standard diet; (BE) standard diet+blackberry anthocyanins extract; (HF) high-fat diet or (HFBE) high-fat diet+blackberry anthocyanins extract group. Values are expressed as mean±SD. Boldface values indicate rank product <0.05.

2.6. RNA isolation and qRT-PCR

Total RNA from treated cells was isolated using RNA-STAT 60 reagent (AMS Biotechnology, UK) according to manufacturer's instructions. Purity of RNA samples was confirmed with absorbance ratios 260/280 nm superior to 1.8. RNA samples were treated with DNase I (RQ1 RNase-free DNase; Promega, Portugal). cDNA was synthesized from 5 μg of treated mRNA with NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal). Quantitative real-time polymerase chain reaction (qRT-PCR) was run on Lightcycler96 (Roche Diagnostics, USA). Ten-microliter reactions were set on 96-well plates using 0.2 μM of each primer and 5 μl of FastStart Essential DNA Green Master (Roche Diagnostics, USA). Cycling conditions were as follows: denaturation (95°C for 10 min), amplification and quantification (95°C for 10 s, annealing temperature for 10 s and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for a 10-s segment) repeated for 55 cycles, and a final melting 95°C for 10 s, 65°C for 60 s and 97°C for 1 s. The following human-specific primers were used: fractalkine (annealing temperature, 63°C) sense 5′ CACCACGGTGTGACGAAATG 3′ and antisense 5′ TCTCCAAGAT GATTGCGCGT; HPRT-1 (annealing temperature, 59°C) sense 5′ TGCTGACCTGCTGGATTACA 3′ and antisense 5′ TTTATGTCCCCTGTT GACTGG 3′. Data were analyzed using LightCycler96 SW analysis Software v1.1. The Cq values obtained were transformed into relative quantification data using the 2^(−ΔCq) [37].

2.7. Data analysis

Results from cortex and plasma arrays were subjected to background subtraction and normalization for positive controls in each array, to allow comparison between arrays. Results are expressed as % expression of a group comparatively to another (mean of duplicates). Rank product test [38] was used to identify the most significant changes (rank product<0.05) between the molecules screened. Data from body weight, food and energy ingestion, arginase activity, ELISA and quantitative array in hippocampus and from qRT-PCR were analyzed in GraphPad Prism 6.0 Software using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was considered when $P<.05$.

3. Results

3.1. Body weight and food ingestion

Animals started the treatment with similar weights and fed with high-fat chow (HF and HFBE) at the end of treatment groups, and they have increased weights compared to standard-chow-fed animals (C and BE) (see Table 1). Energy intake was slightly higher in HFBE group compared to HF; however, this increase did not represent a significant increase in body weight.

3.2. Brain inflammatory profile

Intake of the high-fat diet induced an increase in the expression of several proteins involved in inflammatory response, relative to standard-chow-fed animals (Table 2). In particular, rank product tests [38] indicated that the most differentially expressed were the

Table 3
Effect of 17 weeks of consumption of blackberry anthocyanin extract (25 mg kg^{-1} body weight) on cytokines expression on brain cortex, in animals fed with standard or high-fat diet.

BE effect on brain — animals fed with standard diet			BE effect on brain — animals fed with high-fat diet		
Protein	BE expression vs. C %	Rank product	Protein	HFBE expression vs. HF %	Rank product
Down-regulated			*Down-regulated*		
CNTF	**62.9±13.1**	**0.004**	**TCK-1**	**48.3±3.7**	**0.001**
IFN-γ	**71.3±3.6**	**0.010**	**CINC-3**	**79.1±1.2**	**0.017**
Leptin	**76.7±21.1**	**0.017**	**PDGF-AA**	**88.2±22.2**	**0.022**
MIP-3α	**77.9±9.4**	**0.036**	**CINC-2α**	**84.5±9.6**	**0.026**
Fas ligand	**77.9±4.3**	**0.038**	**CNTF**	**83.3±17**	**0.028**
MMP-8	**84.8±17.3**	**0.040**	**RAGE**	**87.1±2.4**	**0.031**
IL-2	**78.4±5.7**	**0.042**	**TIMP-1**	**82.3±7.9**	**0.035**
TCK-1	76.6±1.2	0.054	**IL-10**	**88.6±1.7**	**0.042**
β-NGF	79.5±3.9	0.067	CINC-1	88.8±11.1	0.065
CD86	92.0±20.7	0.080	Prolactin R	99.4±5.4	0.086
IL-1α	81.1±4.5	0.111	*Up-regulated*		
MCP-1	84.4±6.4	0.125	**Fractalkine**	**197.0±12.7**	**0.005**
RAGE	87.5±5.7	0.218	**IL-13**	**183.0±42.9**	**0.012**
IL-10	91.2±11.7	0.234	**MMP-8**	**166.0±17.4**	**0.016**
TNF-α	92.8±13.2	0.234	**Leptin**	**195.4±110.5**	**0.017**
CINC-1	91.1±6.2	0.234	**VEGF**	**151.6±11.2**	**0.017**
L-Selectin	92.5±8.2	0.236	**Fas ligand**	**171.3±28**	**0.021**
LIX	92.5±4.8	0.265	**TNF-α**	**450.4±262.4**	**0.028**
GM-CSF	92.5±5.7	0.270	**IL-1β**	**143.0±9.3**	**0.043**
IL-1 R6	91.6±3.1	0.285	LIX	133.3±1.5	0.062
CINC-2α	93.7±4.0	0.312	GM-CSF	133.1±2.1	0.067
CINC-3	96.4±9.0	0.368	IFN-γ	129.9±2.0	0.112
Prolactin R	96.0±5.9	0.381	ICAM-1	129.6±8.9	0.118
Up-regulated			MIP-3α	124.1±3.1	0.157
TIMP-1	**144.8±7.5**	**0.002**	CD86	112.1±22.7	0.202
PDGF-AA	**116.8±6.2**	**0.013**	MCP-1	119.0±8.4	0.218
Agrin	**123.4±9.6**	**0.016**	Activin A	116.8±12.6	0.219
IL-1β	**114.2±70.3**	**0.025**	IL-4	107.6±13.3	0.239
VEGF	**111.6±3.1**	**0.031**	IL-1α	110.8±2.3	0.265
Activin A	**108.2±1.1**	**0.036**	Agrin	107.3±10.5	0.272
Fractalkine	**113.8±35.7**	**0.047**	IL-6	105.8±10	0.304
IL-4	102.4±17.2	0.091	β-NGF	102.5±16.2	0.374
IL-6	100.8±5.0	0.093	IL-1 R6	103.9±8.7	0.394
ICAM-1	100.7±1.8	0.095	IL-2	101.5±10.7	0.420

(C) Standard diet; (BE) standard diet+blackberry anthocyanins extract; (HF) high-fat diet or (HFBE) high-fat diet+blackberry anthocyanins extract group. Values are expressed as mean±SD. Boldface values indicate rank product <0.05.

following: the platelet-derived growth factor PDGF-AA, the ciliary neurotrophic factor CNTF, the cytokine-induced neutrophil chemoattractant CINC-3, the interleukin IL-10, the tissue inhibitor of metalloproteinase TIMP-1 and the receptor for advanced glycation end products RAGE, all of which were observed to be at least 4-fold induced compared to standard chow intake (ratio expression HF vs. C, >400%). As detailed in Table 3, in high-fat diet animals supplemented with the blackberry anthocyanin extract, there was a significant down-regulation in the expression of thymus chemokine TCK-1, CINC-3, PDGF-AA, cytokine-induced neutrophil chemoattractant CINC-2α, CNTF, RAGE, TIMP-1 and IL-10 while fractalkine, IL-13, matrix metalloproteinase MMP-8, leptin, vascular endothelial growth factor VEGF, Fas ligand, TNF-α and IL-1β expression were all up-regulated. When animals were supplemented with blackberry anthocyanins extract on the standard chow diet, the expression of CNTF, interferon IFN-γ, leptin, macrophage inflammatory protein MIP-3α, Fas ligand, MMP-8 and IL-2 were decreased relative to the standard diet alone, while that of TIMP-1, PDGF-AA, agrin, IL-1β, VEGF, activin A and fractalkine were increased. In both standard diets and high-fat diets groups, intake of the anthocyanin extract resulted in a decrease in the expression of TCK-1 (P=.054 BE vs. C; P<.01 HFBE vs. HF) and a significant increase in the levels of fractalkine, IL-1β and VEGF (P<.05).

The consumption of blackberry anthocyanins extract also induced a significant increase in fractalkine expression and a sharp decrease in TCK-1 levels in the hippocampus of both standard-fed and high-fat-fed animals (Table 4). PDGF-AA was also significantly increased by the extract but only in rats fed with standard diet.

Blackberry anthocyanins extract supplementation was not able to significantly change hippocampus expression of cytokines, intercellular adhesion molecule ICAM-1, IFN-γ, IL-1β, IL-4, IL-10, IL-13, MCP-1 and granulocyte monocyte colony stimulating factor GM-CSF. Although the antiinflammatory cytokine IL-13 seemed to be increased by the extract, differences were not statistically significant. CD86, β-nerve growth factor (β-NGF), CINC-1, CINC-2, CINC-3, CNTF, LPS-induced CXC chemokine

Table 4
Effect of 17 weeks of consumption of blackberry anthocyanin extract (25 mg kg^{-1} body weight) on cytokines expression on hippocampus.

	C	BE	HF	HFBE
IL-1β	1740±904	1416±307	1335±618	1627±917
IL-4	31.91±4.2	41.32±12	36.27±14	38.49±19
IL-10	5248±672	5263±816	5312±1972	5471±1743
IL-13	42.17±36	134.3±102	90.32±75	132.1±107
Fractalkine	1163±309[a]	1762±439[b]	1088±436[a]	1983±402[b]
GM-CSF	288.8±186	295.6±143	462.6±430	450.2±196
ICAM	2842±1079	2764±814	2971±1242	3153±1547
IFN-γ	98.3±48	69.4±32	63.7±24	94.8±17
MCP-1	132.3±66	178.2±67	151.2±71	153.0±18
PDGF-AA	2226±369[a]	3375±874[b]	2869±880[a,b]	2066±689[a,b]
TCK-1	1333±1823[a]	69.72±39[b]	1666±2769[a]	78.22±35[b]

(C) Standard diet; (BE) standard diet+blackberry anthocyanins extract; (HF) high-fat diet or (HFBE) high-fat diet+blackberry anthocyanins extract group. Values are expressed as mean±SD. Superscript letters represent statistically significant differences (P<.05).

Table 5
Effect of 17 weeks of blackberry extract consumption (25 mg kg^{-1} body weight per day) on microglia activation markers on cortex of Wistar rats fed with standard diet or high-fat diet.

	C	BE	HF	HFBE
IL-1β (pg/mg protein)	987.3±190	927.2±218	980.3±293	759.6±255
Arginase activity (U/L)	17.30±1.6	17.75±3.8	18.48±3.7	18.81±3.2

(C) Standard diet; (BE) standard diet+blackberry anthocyanins extract; (HF) high-fat diet or (HFBE) high-fat diet+blackberry anthocyanins extract group. Values are expressed as mean±SD. Differences were not statistical significant.

(LIX), L-selectin, prolactin receptor, TIMP-1, TNF-α, VEGF, IL-2, IL-6 and RAGE expression were under limit of detection.

3.3. Cortical microglia activation

Microglial status was determined by assessment of markers of M1 proinflammatory and M2 antiinflammatory state [39]. Neither high-fat diet nor the blackberry anthocyanin extract modulated levels of IL-1β (M1 marker) or levels of arginase 1 (M2 marker) (Table 5).

3.4. Systemic inflammatory markers

Similar to that observed in the cortex, the blackberry anthocyanin extract induced a significant decrease in the plasma levels of the adhesion molecules L-selectin and ICAM-1 and the chemoattractant molecules TCK-1 and MIP-3α, relative to animals fed with standard chow only (Table 6). In addition, blackberry anthocyanin extract significantly increased the expression of RANTES, IL-1β, IL-13 and IL-1rα. The cytokines CNTF, IL-4, IFN-γ, CINC-2α/β, TNF-α and MIP-1α were under the limit of detection.

Table 6
Effect of 17 weeks of consumption of blackberry anthocyanin extract (25 mg kg^{-1} body weight) on cytokines expression on plasma of standard-fed animals.

BE effect on plasma — standard-diet-fed animals		
Protein	BE expression vs. C %	Rank product
Down-regulated		
L-Selectin	**22.9±0.5**	**0.002**
sICAM-1	**25.0±1.2**	**0.008**
TCK-1	**27.5±1.2**	**0.017**
MIP-3α	**36.2±4.1**	**0.038**
MIG	38.5±9.9	0.053
Fractalkine	43.0±1.6	0.068
IL-10	47.7±7.2	0.095
VEGF-A	52.1±2.1	0.146
IL-3	49.8±2.5	0.153
GM-CSF	51.9±7.7	0.166
CINC-3	57.1±8.7	0.181
CINC-1	71.4±2.7	0.319
IL-1α	68.1±24.1	0.321
IL-17	74.9±5.1	0.363
IL-6	75.9±7.4	0.423
LIX	78.2±0.1	0.425
IL-2	90.3±21.3	0.556
IP-10	90.1±7.2	0.578
Up-regulated		
RANTES	**188.5±1.8**	**0.002**
IL-1β	**133.4±2.1**	**0.011**
IL-13	**130.4±39.5**	**0.015**
IL-1rα	**106.5±6.1**	**0.038**
TIMP-1	102.4±1.1	0.057

(C) Standard diet; (BE) standard diet+blackberry anthocyanins extract; (HF) high-fat diet or (HFBE) high-fat diet+blackberry anthocyanins extract group. Values are expressed as mean±SD. Boldface values indicate rank product <0.05.

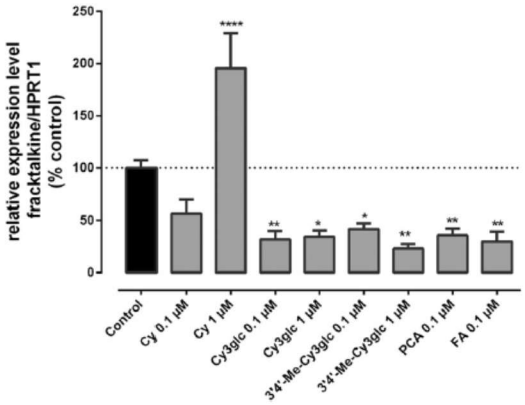

Fig. 1. Effect of cyanidin (Cy), cyanidin-3-glucoside (Cy3glc), 3′ and 4′-Me-Cy3glc (3′ or 4′-methylcyanidin-3-glucoside), PCA and FA on the transcription of fractalkine in SH-SY5Y neurons. mRNA levels were determined by qRT-PCR and normalized to the housekeeping gene (hypoxanthine-guanine-phosphoribosyltransferase, HPRT). Values are expressed as mean±standard error of mean of duplicated samples from four independent experiments. Statistical analysis was performed with two-way ANOVA, followed by Dunnet's multiple comparison test: *$P<.05$; **$P<.01$; ***$P<.001$.

3.5. Fractalkine mRNA expression in an in vitro model

To assess the potential of anthocyanins to modulate fractalkine transcription in neurons, cyanidin-3-glucoside (Cy3glc; the main anthocyanin present in the blackberry anthocyanin extract), its aglycone cyanidin (Cy), one of its phase I/II metabolites (3′4′-Me-Cy3glc) and the bacterial metabolites protocatechuic acid (PCA) and ferulic acid (FA) were tested in the neuron-like cell line SH-SY5Y.

As shown in Fig. 1, Cy (1 μM) increased fractalkine expression (relative increase of 96%) relative to vehicle treated cells. In contrast, Cy3glc (0.1 μM and 1 μM), 3′4′-Me-Cy3glc (0.1 μM and 1 μM), PCA (0.1 μM) and ferulic acid (0.1 μM) all induced a reduction in fractalkine expression (Fig. 1).

4. Discussion

Dietary intervention with blackberry has previously been shown to be effective in modulating antioxidant enzyme expression and markers of oxidative status in rodents [40]. Furthermore, flavonoids have been shown to inhibit neuroinflammation by attenuating cytokines release (IL-1β and TNF-α), nitric oxide production and activity in NADPH oxidase activity in cellular systems [41,42]. Such effects are postulated to underpin blackberry-induced behavioral and motor improvements, in rats [43]. The present study aimed to assess the impact of a chronic blackberry anthocyanin extract supplementation on the neuroinflammatory profile in both cortex and hippocampus of rats fed with either a standard or a high-fat diet (45% fat). High-fat-fed animals clearly developed an excessive weight increase not reverted by BE supplementation. The dose given to rats — 25 mg/body weight — would correspond to approximately 243 mg blackberry extract in a human adult with 60 kg [44] that could be easily achieved by diet eating as much as 100 g blackberries a day [45]. Results obtained in this study went further than showing the potential of anthocyanins to counteract diet-induced inflammation but also suggested that, without an inflammatory context, anthocyanins are able to modulate processes involved in synaptic plasticity.

The expression of several proteins was increased in the cortex of high-fat-fed animals relative to standard-chow-fed animals, supporting previous observations that the high intake of fat may contribute to brain inflammation [9,46]. Concurrent supplementation of BE in high-fat-fed animals led to a down-regulation of CINC-3, CNTF, IL-10 and

RAGE, all increased by high-fat diet. The inhibition of RAGE, which has a central role in the inflammatory response [47,48] and CINC-3 expression following blackberry anthocyanin intake, is likely to result in a decreased rate of inflammatory propagation [49]. In support of this, *in vitro* evidence also showed that anthocyanins decreased the expression of RAGE [50]. Not so clear is the relevance of increased cortical expression of TNF-α and IL-1β with BE in high-fat challenged animals. Although these cytokines are recognized mainly as proinflammatory, recent studies suggested their involvement in hippocampus neurogenesis and neuronal differentiation [51–53].

The blackberry extract also modulated several proteins involved in synaptic plasticity, with PDGF-AA and fractalkine [54,55] consistently modulated by BE in the cortex and the hippocampus of standard-fed animals. Moreover, the BE also increased cortex expression of activin A, agrin and VEGF, all known to be important in synapse structure, regeneration and synaptogenesis [56–58]. Together, our data suggest a role for anthocyanins, delivered through blackberry, in regulating synaptic plasticity [54–60]. Increases in TIMP-1 expression and decreases in MMP-8 in the cortex of standard-fed animals are consistent with this hypothesis, since many of MMP substrates are involved in synaptogenesis, synaptic plasticity and long-term potentiation [61]. On the other hand, the effects of the anthocyanin extract in a presence of a high-fat diet were more variable, with MMP-8 up-regulated, TIMP-1 down-regulated and no effects on activin A and agrin observed. Thus, the potential to modulate synaptic plasticity of the blackberry intervention seems to be lessened (and perhaps worsened) in the presence of a high-fat challenged brain where neuroinflammation is developed. However, as we discussed above, flavonoids seemed to have a beneficial role modulating inflammatory mediators in these high-fat challenged brains.

The modulation of fractalkine expression by BE was one of the most consistent findings, since it was increased in both cortex and hippocampus, independent of fat diet content. Fractalkine is a chemokine synthesized by neurons, whose receptor is highly expressed in microglia cells [62]. Fractalkine and its receptor represent an important axis in neuronal/microglial crosstalk and this axis may be essential in maintaining microglia in a resting state in the healthy brain as well as preventing excessive microglial activation during inflammatory conditions [63,64]. Previous data suggest that flavonoids are capable of changing the phenotype of microglia by restoring the population of microglial cells to the "resting" state from the more active state [65]. However, despite observing consistent increases in the expression of fractalkine, and other proteins related to synaptic plasticity, blackberry anthocyanin extract intake did not result in a significant change in the phenotype of activation (M1/M2) of microglia, as evidenced by no significant modulation of IL-1β and arginase activity, respectively.

Hippocampal chemokine TCK-1 expression was also strongly modulated by the blackberry anthocyanin intake. As genomic analysis indicates significant sequence similarity between TCK-1 and fractalkine [66], we suggest that their expression may be controlled by a similar mechanism. To further explore the ability of BE to modulate fractalkine expression, anthocyanins and their physiological metabolites were assessed *in vitro* for their potential to influence neuronal fractalkine mRNA expression. Cyanidin markedly increased the expression of fractalkine at the concentration of 1 μM, while cyanidin-3-glucoside and metabolites induced a significant reduction on fractalkine mRNA levels. Not disclosing the possibility of tissue accumulation of anthocyanin previously suggested by Kalt and colleagues [67], concentrations of 0.1 μM are more likely to be representative of concentrations found in circulation and consequently in brain [68–70]. Despite the disparity between our *in vitro* and *in vivo* models, the *in vitro* data confirm the potential of flavonoid aglycones to affect fractalkine expression. Nevertheless, *in vitro* models lack the complexity of the CNS, notably here the absence of microglia and astrocytes that may be required for the optimal regulation of inflammatory proteins.

It is now accepted that neuroinflammation is not an isolated process and peripheral stimulus can directly or indirectly propagated to the brain [71,72]. In the present study, chemokines regulation in the plasma was observed, with the blackberry extract decreasing chemokines MIP-3α and TCK-1 and adhesion molecules ICAM and L-selectin expression in standard-chow-fed animals. The latter are associated with vascular inflammation and regulate leucocyte trafficking in the vessel wall [73]. In the context of the brain, reductions in ICAM-1 by BE may limit leucocyte migration across BBB, as well as in endothelial cells [74]. Previous studies have already indicated that blackberry anthocyanins were protective against endothelial dysfunction [75] and capable of modulate ICAM-1 expression [76]. Our findings showed that the blackberry anthocyanin extract also had effects on periphery; however, it is not clear to which extend it affects changes observed in cortex and hippocampus.

In summary, our data provide further insight into the potential benefits of long-term anthocyanin-rich food ingestion. Under low-fat conditions, chronic consumption of blackberry anthocyanin extract specifically and beneficially regulated PDGF-AA, fractalkine, activin, VEGF and agrin, which together may aid synapse connectivity. Under high-fat conditions, where there was extensive regulation of inflammatory proteins, BE intake partly attenuated the negative impact of fat intake. As high-fat intake may lead to earlier onset of dementia [77,78], blackberry and other anthocyanin-rich food intake may be capable of preventing the detrimental effects of neuroinflammation in a high-fat challenged brain. Lastly, we suggest that fractalkine and TCK-1 expression may be specific targets of anthocyanins and their metabolites within the CNS, although further work with microglia or astrocytes cocultures or their conditioned medium would be helpful to fully explore this issue.

Abbreviations

CD86	cluster of differentiation 86
CINC	cytokine-induced neutrophil chemoattractant
CNTF	ciliary neurotrophic factor
FA	ferulic acid
GM-CSF	granulocyte monocyte colony stimulating factor
ICAM	intercellular adhesion molecule
IFN	interferon γ
IL	interleukin
LIX	LPS-induced CXC chemokine
MCP-1	macrophage chemoattractant protein 1
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
PDGF-AA	platelet-derived growth factor AA
PCA	protocatechuic acid
Prolactin R	prolactin receptor
RAGE	receptor for advanced glycation end products
TCK-1	thymus chemokine 1
TIMP-1	tissue inhibitor of metalloproteinase 1
TNF-α	tumor necrosis factor α
VEGF	vascular endothelial growth factor
β-NGF	β-nerve growth factor

Author's Contributions

MM contributed for the experimental design, performed the experiments, analyzed and interpreted data and prepared the manuscript.

CM and SN helped with animal work and data interpretation. IF and NM were responsible for the preparation of blackberry extract and metabolites synthesis. CR contributed for data acquisition and improvements in the draft manuscript. AF and CC were responsible for study design and with JPES for overall experiments conduction, data analyses and interpretation and critically revision of manuscript.

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References

[1] Organization WH, International AsD. Dementia: a public health priority. World Health Organization Publications; 2012.
[2] Amor S, Puentes F, Baker D, van der Valk P. Inflammation in neurodegenerative diseases. Immunology 2010;129:154–69.
[3] Barcia C, Hunot S, Guillemin GJ, Pitossi F. Inflammation and Parkinson's disease. Park Dis 2011;2011:729054.
[4] McGeer PL, McGeer EG. NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. Neurobiol Aging 2007;28:639–47.
[5] Vlad SC, Miller DR, Kowall NW, Felson DT. Protective effects of NSAIDs on the development of Alzheimer disease. Neurology 2008;70:1672–7.
[6] Cai D. Neuroinflammation and neurodegeneration in overnutrition-induced diseases. Trends Endocrinol Metab 2013;24:40–7.
[7] Vafeiadou K, Vauzour D, Spencer JP. Neuroinflammation and its modulation by flavonoids. Endocr Metab Immune Disord Drug Targets 2007;7:211–24.
[8] Frisardi V, Solfrizzi V, Seripa D, Capurso C, Santamato A, Sancarlo D, et al. Metabolic-cognitive syndrome: a cross-talk between metabolic syndrome and Alzheimer's disease. Ageing Res Rev 2010;9:399–417.
[9] Jayaraman A, Lent-Schochet D, Pike CJ. Diet-induced obesity and low testosterone increase neuroinflammation and impair neural function. J Neuroinflammation 2014;11:162–75.
[10] Gardener SL, Rainey-Smith SR, Barnes MB, Sohrabi HR, Weinborn M, Lim YY, et al. Dietary patterns and cognitive decline in an Australian study of ageing. Mol Psychiatry 2014.
[11] Kanoski SE, Davidson TL. Western diet consumption and cognitive impairment: links to hippocampal dysfunction and obesity. Physiol Behav 2011;103:59–68.
[12] Pistell PJ, Morrison CD, Gupta S, Knight AG, Keller JN, Ingram DK, et al. Cognitive impairment following high fat diet consumption is associated with brain inflammation. J Neuroimmunol 2010;219:25–32.
[13] Zhang X, Dong F, Ren J, Driscoll MJ, Culver B. High dietary fat induces NADPH oxidase-associated oxidative stress and inflammation in rat cerebral cortex. Exp Neurol 2005;191:318–25.
[14] Thirumangalakudi L, Prakasam A, Zhang R, Bimonte-Nelson H, Sambamurti K, Kindy MS, et al. High cholesterol-induced neuroinflammation and amyloid precursor protein processing correlate with loss of working memory in mice. J Neurochem 2008;106:475–85.
[15] Spencer JP. Flavonoids and brain health: multiple effects underpinned by common mechanisms. Genes Nutr 2009;4:243–50.
[16] Youdim KA, Spencer JP, Schroeter H, Rice-Evans C. Dietary flavonoids as potential neuroprotectants. Biol Chem 2002;383:503–19.
[17] Letenneur L, Proust-Lima C, Le Gouge A, Dartigues JF, Barberger-Gateau P. Flavonoid intake and cognitive decline over a 10-year period. Am J Epidemiol 2007;165:1364–71.
[18] Engelhart MJ, Geerlings MI, Ruitenberg A, van Swieten JC, Hofman A, Witteman JC, et al. Dietary intake of antioxidants and risk of Alzheimer disease. JAMA 2002;287:3223–9.
[19] Gao X, Cassidy A, Schwarzschild MA, Rimm EB, Ascherio A. Habitual intake of dietary flavonoids and risk of Parkinson disease. Neurology 2012;78:1138–45.
[20] Spencer JP, Vafeiadou K, Williams RJ, Vauzour D. Neuroinflammation: modulation by flavonoids and mechanisms of action. Mol Asp Med 2012;33:83–97.
[21] Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci 2004;96:229–45.
[22] Norberto S, Silva S, Meireles M, Faria A, Pintado M, Calhau C. Blueberry anthocyanins in health promotion: A metabolic overview. J Funct Foods 2013;5:1518–28.
[23] Williams CM, El Mohsen MA, Vauzour D, Rendeiro C, Butler LT, Ellis JA, et al. Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neurotrophic factor (BDNF) levels. Free Radic Biol Med 2008;45:295–305.
[24] Vafeiadou K, Vauzour D, Lee HY, Rodriguez-Mateos A, Williams RJ, Spencer JP. The citrus flavanone naringenin inhibits inflammatory signalling in glial cells and protects against neuroinflammatory injury. Arch Biochem Biophys 2009;484:100–9.
[25] Jang S, Dilger RN, Johnson RW. Luteolin inhibits microglia and alters hippocampal-dependent spatial working memory in aged mice. J Nutr 2010;140:1892–8.
[26] Wang YJ, Thomas P, Zhong JH, Bi FF, Kosaraju S, Pollard A, et al. Consumption of grape seed extract prevents amyloid-beta deposition and attenuates inflammation in brain of an Alzheimer's disease mouse. Neurotox Res 2009;15:3–14.
[27] Hwang IK, Lee CH, Yoo KY, Choi JH, Park OK, Lim SS, et al. Neuroprotective effects of onion extract and quercetin against ischemic neuronal damage in the gerbil hippocampus. J Med Food 2009;12:990–5.
[28] Xu Z, Chen S, Li X, Luo G, Li L, Le W. Neuroprotective effects of (-)-epigallocatechin-3-gallate in a transgenic mouse model of amyotrophic lateral sclerosis. Neurochem Res 2006;31:1263–9.
[29] Joseph JA, Shukitt-Hale B, Willis LM. Grape juice, berries, and walnuts affect brain aging and behavior. J Nutr 2009;139:1813S–7S.
[30] Poulose SM, Carey AN, Shukitt-Hale B. Improving brain signaling in aging: Could berries be the answer? Expert Rev Neurother 2012;12:887–9.
[31] Azevedo J, Fernandes I, Faria A, Oliveira J, Fernandes A, de Freitas V, et al. Antioxidant properties of anthocyanidins, anthocyanidin-3-glucosides and respective portisins. Food Chem 2010;119:518–23.
[32] Oliveira J, Fernandes V, Miranda C, Santos-Buelga C, Silva A, de Freitas V, et al. Color properties of four cyanidin-pyruvic acid adducts. J Agric Food Chem 2006;54:6894–903.
[33] Fernandes I, Azevedo J, Faria A, Calhau C, de Freitas V, Mateus N. Enzymatic hemisynthesis of metabolites and conjugates of anthocyanins. J Agric Food Chem 2009;57:735–45.
[34] Fernandes I, Marques F, de Freitas V, Mateus N. Antioxidant and antiproliferative properties of methylated metabolites of anthocyanins. Food Chem 2013;141:2923–33.
[35] Cherry JD, Olschowka JA, O'Banion MK. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J Neuroinflammation 2014;11:98–112.
[36] Tarozzi A, Morroni F, Hrelia S, Angeloni C, Marchesi A, Cantelli-Forti G, et al. Neuroprotective effects of anthocyanins and their in vivo metabolites in SH-SY5Y cells. Neurosci Lett 2007;424:36–40.
[37] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008;3:1101–8.
[38] Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett 2004;573:83–92.
[39] Boche D, Perry VH, Nicoll JA. Review: activation patterns of microglia and their identification in the human brain. Neuropathol Appl Neurobiol 2013;39:3–18.
[40] Hassimotto NM, Lajolo FM. Antioxidant status in rats after long-term intake of anthocyanins and ellagitannins from blackberries. J Sci Food Agric 2011;91:523–31.
[41] Vauzour D, Vafeiadou K, Rodriguez-Mateos A, Rendeiro C, Spencer JP. The neuroprotective potential of flavonoids: a multiplicity of effects. Genes Nutr 2008;3:115–26.
[42] Johnson MH, de Mejia EG, Fan J, Lila MA, Yousef GG. Anthocyanins and proanthocyanidins from blueberry-blackberry fermented beverages inhibit markers of inflammation in macrophages and carbohydrate-utilizing enzymes in vitro. Mol Nutr Food Res 2013;57:1182–97.
[43] Shukitt-Hale B, Cheng V, Joseph JA. Effects of blackberries on motor and cognitive function in aged rats. Nutr Neurosci 2009;12:135–40.
[44] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J 2008;22:659–61.
[45] Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. J Agric Food Chem 2006;54:4069–75.
[46] Nerurkar PV, Johns LM, Buesa LM, Kipyakwai G, Volper E, Sato R, et al. Momordica charantia (bitter melon) attenuates high-fat diet-associated oxidative stress and neuroinflammation. J Neuroinflammation 2011;8:64–82.
[47] Chavakis T, Bierhaus A, Nawroth PP. RAGE (receptor for advanced glycation end products): a central player in the inflammatory response. Microbes Infect 2004;6:1219–25.
[48] Fang F, Lue LF, Yan S, Xu H, Luddy JS, Chen D, et al. RAGE-dependent signaling in microglia contributes to neuroinflammation, Abeta accumulation, and impaired learning/memory in a mouse model of Alzheimer's disease. FASEB J 2010;24:1043–55.
[49] Ubogu EE, Cossoy MB, Ransohoff RM. The expression and function of chemokines involved in CNS inflammation. Trends Pharmacol Sci 2006;27:48–55.
[50] Lu J, Wu DM, Zheng YL, Hu B, Zhang ZF. Purple sweet potato color alleviates D-galactose-induced brain aging in old mice by promoting survival of neurons via PI3K pathway and inhibiting cytochrome C-mediated apoptosis. Brain Pathol 2010;20:598–612.
[51] Seguin JA, Brennan J, Mangano E, Hayley S. Proinflammatory cytokines differentially influence adult hippocampal cell proliferation depending upon the route and chronicity of administration. Neuropsychiatr Dis Treat 2009;5:5–14.
[52] Peng H, Whitney N, Wu Y, Tian C, Dou H, Zhou Y, et al. HIV-1-infected and/or immune-activated macrophage-secreted TNF-alpha affects human fetal cortical neural progenitor cell proliferation and differentiation. Glia 2008;56:903–16.
[53] Conner C, Ackerman KM, Lahne M, Hobgood JS, Hyde DR. Repressing notch signaling and expressing TNFalpha are sufficient to mimic retinal regeneration by

inducing Muller glial proliferation to generate committed progenitor cells. J Neurosci 2014;34:14403–19.
[54] Valenzuela CF, Kazlauskas A, Weiner JL. Roles of platelet-derived growth factor in the developing and mature nervous systems. Brain Res Brain Res Rev 1997;24: 77–89.
[55] Rogers JT, Morganti JM, Bachstetter AD, Hudson CE, Peters MM, Grimmig BA, et al. CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. J Neurosci 2011;31:16241–50.
[56] Shoji-Kasai Y, Ageta H, Hasegawa Y, Tsuchida K, Sugino H, Inokuchi K. Activin increases the number of synaptic contacts and the length of dendritic spine necks by modulating spinal actin dynamics. J Cell Sci 2007;120:3830–7.
[57] Daniels MP. The role of agrin in synaptic development, plasticity and signaling in the central nervous system. Neurochem Int 2012;61:848–53.
[58] Rosenstein JM, Krum JM, Ruhrberg C. VEGF in the nervous system. Organogenesis 2010;6:107–14.
[59] Deyst KA, Ma J, Fallon JR. Agrin: toward a molecular understanding of synapse regeneration. Neurosurgery 1995;37:71–7.
[60] Funa K, Sasahara M. The roles of PDGF in development and during neurogenesis in the normal and diseased nervous system. J Neuroimmune Pharmacol 2014;9:168–81.
[61] Ethell IM, Ethell DW. Matrix metalloproteinases in brain development and remodeling: synaptic functions and targets. J Neurosci Res 2007;85:2813–23.
[62] Kettenmann H, Kirchhoff F, Verkhratsky A. Microglia: new roles for the synaptic stripper. Neuron 2013;77:10–8.
[63] Wolf Y, Yona S, Kim KW, Jung S. Microglia, seen from the CX3CR1 angle. Front Cell Neurosci 2013;7:26–34.
[64] Jurgens HA, Johnson RW. Dysregulated neuronal-microglial cross-talk during aging, stress and inflammation. Exp Neurol 2012;233:40–8.
[65] Jang S, Johnson RW. Can consuming flavonoids restore old microglia to their youthful state? Nutr Rev 2010;68:719–28.
[66] Hiroyama T, Iwama A, Nakamura Y, Nakauchi H. Fractalkine shares signal sequence with TARC: gene structures and expression profiles of two chemokine genes. Genomics 2001;75:3–5.
[67] Kalt W, Blumberg JB, McDonald JE, Vinqvist-Tymchuk MR, Fillmore SAE, Graf BA, et al. Identification of Anthocyanins in the Liver, Eye, and Brain of Blueberry-Fed Pigs. J Agric Food Chem 2008;56:705–12.
[68] Czank C, Cassidy A, Zhang Q, Morrison DJ, Preston T, Kroon PA, et al. Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a (13)C-tracer study. Am J Clin Nutr 2013;97:995–1003.
[69] Tsuda T, Horio F, Osawa T. Absorption and metabolism of cyanidin 3-O-beta-D-glucoside in rats. FEBS Lett 1999;449:179–82.
[70] Talavéra S, Felgines C, Texier O, Besson C, Gil-Izquierdo A, Lamaison J-L, et al. Anthocyanin Metabolism in Rats and Their Distribution to Digestive Area, Kidney, and Brain. J Agric Food Chem 2005;53:3902–8.
[71] Lyman M, Lloyd DG, Ji X, Vizcaychipi MP, Ma D. Neuroinflammation: the role and consequences. Neurosci Res 2014;79:1–12.
[72] Fung A, Vizcaychipi M, Lloyd D, Wan Y, Ma D. Central nervous system inflammation in disease related conditions: mechanistic prospects. Brain Res 2012;1446:144–55.
[73] Witkowska AM. Soluble ICAM-1: a marker of vascular inflammation and lifestyle. Cytokine 2005;31:127–34.
[74] Greenwood J, Etienne-Manneville S, Adamson P, Couraud PO. Lymphocyte migration into the central nervous system: implication of ICAM-1 signalling at the blood-brain barrier. Vasc Pharmacol 2002;38:315–22.
[75] Serraino I, Dugo L, Dugo P, Mondello L, Mazzon E, Dugo G, et al. Protective effects of cyanidin-3-O-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure. Life Sci 2003;73:1097–114.
[76] Rossi A, Serraino I, Dugo P, Di Paola R, Mondello L, Genovese T, et al. Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation. Free Radic Res 2003;37:891–900.
[77] Kalmijn S, Launer LJ, Ott A, Witteman JC, Hofman A, Breteler MM. Dietary fat intake and the risk of incident dementia in the Rotterdam Study. Ann Neurol 1997;42:776–82.
[78] Luchsinger JA, Tang M, Shea S, Mayeux R. CAloric intake and the risk of alzheimer disease. Arch Neurol 2002;59:1258–63.

CHAPTER VI

Anthocyanin effects on microglia M1/M2 phenotype: consequence on neuronal differentiation and fractalkine expression

Anthocyanin effects on microglia M1/M2 phenotype: consequence on neuronal differentiation and fractalkine expression

Manuela Meireles[1], Cláudia Marques[1], Sónia Norberto[1,2], Paulo Santos[3], Iva Fernandes[4], Nuno Mateus[4], Ana Faria[1,4,5], Conceição Calhau[1,2]*

1- Department of Biochemistry, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal
2-CINTESIS - Center for Research in Health Technologies and Information Systems, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal
3- Life Sciences Department, University of Coimbra, 3001-401 Coimbra, Portugal
4-REQUIMTE/LAQV, Faculty of Sciences, University of Porto, 4169-009 Porto, Portugal
5-Faculty of Nutrition and Food Sciences, University of Porto, 4200-465 Porto, Portugal

Abstract

Microglia mediate multiple aspects of neuroinflammation, including cytotoxicity, repair, regeneration, and immunosuppression due to their ability to acquire diverse activation states, or phenotypes. Modulation of microglial phenotype or microglia-neuron crosstalk can be an appealing neurotherapeutic strategy. Anthocyanins are a class of flavonoids found e.g. in berries that has been attracting interest due to its neuroprotective potential. N9 microglia cell line was treated with 1 μM cyanidin (Cy), cyanidin-3-glucose (Cy3glc) and a methylated form of cyanidin-3-glucose (Met-Cy3glc) in basal conditions and with LPS/IL-4 stimulation. SH-SY5Y cell line was treated with the conditioned medium of microglia and with the anthocyanins alone.

At basal conditions, treatment with anthocyanins for 24 h induced a less pro-inflammatory profile. Decreased TNF-α mRNA expression was induced either by Cy and Met-Cy3glc. LPS markedly increase IL-6 mRNA expression, which was lowered by Cy3glc. IL-1β LPS-induced expression was reverted by Cy. Cy increased CX3CL1 mRNA expression in SH-SY5Y comparing either with control or LPS.

Anthocyanins and metabolites were not able to shift microglia to an M2 strict phenotype however they did interact with microglia biology. There was an attenuation of M1 phenotype and increase of neuronal expression of CX3CL1. Understanding how flavonoids interact with microglia-neuron crosstalk can open new directions for future flavonoids interventions.

*Corresponding author:
Conceição Calhau
Department of Biochemistry
Faculty of Medicine of the University of Porto
Al. Prof. Hernâni Monteiro
4200-319 Porto – Portugal
Email: ccalhau@med.up.pt
Phone/Fax: +351 22 551 36 24

1-Introduction

In the last years there has been a growing interest on the role that microglia plays within the brain. Microglia were well described as the immune effector cells crucial in response to injury in central nervous system (CNS), generating and maintaining inflammatory responses (1). Localized inflammation in CNS has been proved important in the initiation and progress of many neurodegenerative diseases. Still, the relevance of investigating microglia is no longer restricted to neuroinflammation and neurodegenerative diseases (2). Knowledge that microglia are able of acquiring diverse phenotypes in response to environmental stimulus is shifting the perspective of microglia role in the brain (3). Similar to what was previous described for macrophages in periphery, microglia phenotype may be determinant in their functions (4). Microglia phenotype is still a matter of debate, however four activation states have been proposed: the classical M1 activated state; M2a, an alternative activation state involved in repair and regeneration; M2b, an immunoregulatory phenotype and M2c, an acquired deactivating phenotype (5). M1 classical activation is induced by microbial compounds (LPS) or pro-inflammatory cytokines such as interferon-γ (IFN-γ). TNF-α, IL1-β, IL-6, iNOS are typical hallmarks of this classic M1 phenotype. In contrast, IL-4 and IL-10 have shown to differentiate microglia towards an M2a and M2c phenotype respectively, while M2b phenotype is triggered by immune complexes. Arginase I, the

chitinase-like 3 (YM1), FIZZ 1, CCR2 are common molecular markers of M2 alternative activation. The impact of switching in neuronal function is still not completely unveiled, but an M2 phenotype is more likely to be related with synaptic pruning and synaptic plasticity, functions recently attributed to microglia (6, 7). One way neurons and microglia use to communicate is by ligand-receptor axis as CX3CL1/CX3CR1. CX3CL1, also known as fractalkine, is expressed in neuronal cells, binds to and activates CX3CR1 receptors on microglia (8, 9). Modulation of microglial phenotype or microglia-neuron crosstalk could be an appealing neurotherapeutic strategy.

Flavonoids are a class of polyphenols found in fruits and vegetables. In the last decade epidemiological studies have shown the ability of flavonoids to prevent the decline of cognitive function characteristic of ageing and also to improve several memory dependent tasks (10, 11). Several possible mechanisms are pointed for flavonoids action, such as antioxidant potential, ability to interact with signalling cascades, induce vascularization, modulation of nutrients or neurotransmitter transport and ability to counteract neuroinflammation (12-15). Flavonoids have been pointed as inhibitors of microglia activation and counteract release of some pro-inflammatory cytokines as TNF-α and IL-1β (16). However as the concept of microglia and its function is changing, expanding this knowledge is compulsory. If flavonoids are, in fact, able to keep microglia in a senescent state as previously suggested, the question is if they would also induce a M2 prone state (17-19). Future therapeutic approaches targeting cerebral inflammation may shift from suppress of all microglia activation to subtly modify the balance between different phenotypes [19].

In this work we aimed to analyse whether flavonoid treatment could affect microglia M1 or M2 phenotype. Also, as microglia has been implicated in modulation of synaptic structures, the effect on differentiation or expression of synaptic-related molecules in neurons will be evaluated.

2-Methods

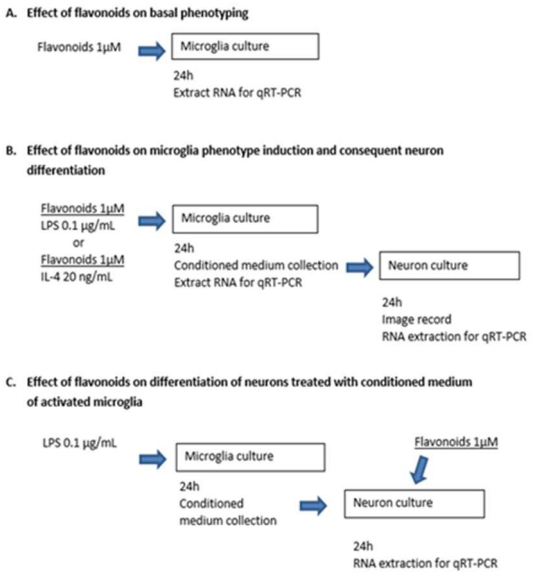
2.1- Cell cultures

As model for microglia, studies were performed with the microglia cell line N9. This cell line (firstly developed by Righi M 1995) was kindly provided by Claudia Verderio, from National Research Council, Milan, Italy. Cells were grown in RPMI 1640 medium (Sigma-Aldrich Chemicals®,Madrid, Spain) containing 5% fetal bovine serum, 25 mM glucose, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Sigma) and passaged twice a week. Cell lines were maintained in a humidified atmosphere of 5% CO2–95% air at 37°C. The neuroblastoma cell line SH-SY5Y, used as undiffentiated neuron model, was grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM-F12Ham, Sigma-Aldrich Chemicals®,Madrid, Spain), supplemented with 1.19 g/L NaHCO3, 3.47 g/L HEPES, 5% fetal bovine serum, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (all from Sigma). Culture medium was changed every 2–3 days and the culture was split every 7 days.

2.2- Treatment conditions and microglia stimulation

Microglia cells were seeded at a density of 3x104 cells per well in six-well plates. After 24 hours cells were treated: (i) with either 1 µM cyanidin (Cy), cyanidin-3-glucose (Cy3glc), a mixture of 3'-methyl-cyanidin and 4'-methyl-cyanidin (Met-cy3glc) or vehicle alone for more 24 hours (figure 1A); (ii) with lipopolysaccharide (LPS from Escherichia coli 011:B4, L4391, Sigma-Aldrich Chemicals®,Madrid, Spain) or IL-4 mouse recombinant (Prepotech, London, UK) and simultaneously with either 1 µM cy, cy3gl, met-cy3glc or vehicle alone for more 24 hours (figure 1B).

Figure 1- Schematic representation of the experimental setups



SH-SY5Y was treated with microglia conditioned medium (CM) of the several conditions described in (ii). Also, SH-SY5Y were cultured

Table 1- Sequence of primers used and respective annealing temperature

N9	Forward	Reverse	T (ºC)
HPRT1	5′ TGCTGACCTGCTGGATTACA 3′	5′ TTTATGTCCCCTGTTGACTGG 3′	59
TNF-α	5′ GATCGGTCCCCAAAGGGATG 3′	5′ TGAGGGTCTGGGCCATAGAA 3′	63
IL-6	5′ GTTCTCTGGGAAATCGTGGA 3′	5′ TTCTGCAAGTGCATCATCGT 3′	59
iNOS	5′ GACGAGACGGATAGGCAGAG 3′	5′ GTGGGGTTGTTGCTGAACTT3′	59
Arg1	5′ AAAGCTGGTCTGCTGGAAAA 3′	5′ ACAGACCGTGGGTTCTTCAC 3′	59
Ym1	5′ CAGTGGCTCAAGGACAACAA 3′	5′ TCTCTGGTGACAGAAAGAACCA 3′	59
CCR2	5′ CTCAGTTCATCCACGGCATAC 3′	5′ GACAAGGCTCACCATCATCG 3′	60
Cyc1	5′ TCAACCCTTACTTTCCCGGC 3′	5′ CCCATGCGTTTTCGATGGTC 3′	63
CX3CR1	5′ CTTTGGGGGCATATTCTTCA 3′	5′ ACGCCCAGACTAATGGTGAC 3′	59
SH-SY5Y			
CX3CL1	5′ CACCACGGTGTGACGAAATG 3′	5′ TCTCCAAGATGATTGCGCGT 3′	63
Eif4a2	5′ CGGTTTTGAGAAGCCTTCCG 3′	5′ GCCAGTACCTGACTGAGCTT 3′	60
Synapsin	5′ AAATATGACGTGCGTGTCCA 3′	5′ TGGTCTTCCAGTTCCCTGAC 3′	59

with conditioned medium of LPS activated microglia and simultaneously treated with cy, cy3gl, met-cy3glc or vehicle alone (figure 1C).
For studies involving CM, medium was collected from the microglia after activation, centrifuged 350 g for 5 minutes and the supernatant (1.5 ml) was used (20). SH-SY5Y medium was replaced for CM for 24 hours.

2.3- RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from treated cells was isolated using RNA-STAT 60 reagent (AMS Biotechnology, UK) according with manufacturer's instructions. Purity of RNA samples was confirmed by absorbance ratio 260/280 nm higher than 1.8. RNA samples were treated with DNaseI (RQ1 RNase-free DNase, Promega, Portugal). cDNA was synthetized from 1 µg of treated mRNA with NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal). Quantitative real-time polymerase chain reaction (qRT-PCR) was run on Lightcycler®96 (Roche Diagnostics, USA). Ten microliter reactions were set on 96 well plates using 0.2 µM of each primer and 5 µL of FastStart Essential DNA Green Master (Roche Diagnostics, USA). Cycling conditions were as follows: denaturation (95°C for 10 min), amplification and quantification (95°C for 10 s, annealing temperature (AT- see table 1) for 10 s, and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment) repeated for 55 cycles, and a final melting 95°C 10 sec, 65°C 60 sec and 97°C for 1 sec. The specific primers sequence used are described on table 1. Data were analysed using LightCycler® 96 SW analysis Software v1.1. Results obtained were normalized for two housekeeping genes (hypoxanthine-guanine-phosphoribosyltransferase- HPRT- and cytochrome c1- Cyc1- for N9 cells; HPRT and eukaryotic translation initiation factor 4A2 for SH-SY5Y cells) by the 2-ΔΔCt method (20-22).

2.4- Differentiation studies

SH-SY5Y cells were seeded at a density of 2 x 105 cells/well in a six-well plate and cultured for 24 hours. The medium was then replaced with the CM of microglia for more 24 hours. Digital images of five randomly selected fields were acquired with an optical microscope (Nikon Eclipse 50i®, Melville, USA), at a magnification of 20x. Cells bearing neurite-like processes longer than the diameter of the cell body were counted. Following previous literature (23) the percentage of differentiation was calculated from the number of cells that showed process outgrowth divided by the total number of cells in each field.

2.5-Statistical analyses

Values are expressed as the arithmetic mean (SEM) of duplicated samples from four independent experiments. Statistical significance of the difference between various groups was evaluated with GrahPad Prism® 6.0 Software by one-way analysis of variance (ANOVA) followed by Fisher's LSD multiple comparison test. Differences were considered significant when $p < 0.05$.

3-Results

3.1- Effect of cyanidin-3-glucose and derivatives on microglia basal phenotype

The first goal of this work was to measure the anthocyanin direct effects on microglia polarization. N9 microglia cells were treated with cyanidin (Cy), cyanidin-3-glucose (Cy3glc)

and a mixture of 3' and 4'-methylcyanidin-3-glucose (Met-cy3glc) for 24 hours. mRNA expression of IL-6, iNOS, TNF-α (specific markers of M1), Ym1, CCR2 and Arg1 (specific markers of M2) and CX3CR1 also associated with M2 were analysed. TNF-α mRNA expression was consistently lowered by all the conditions tested although for Cy3glc without statistical significance (Table 2).

Table 2- Effect of 1 µM of cyanidin (Cy), cyanidin-3-glucose (Cy3glc), 3' and 4'-Met-Cy3glc (Met-Cy3glc), on the transcription of microglia markers in N9 microglia cell line.

	Basal		
	Cy	Cy3glc	Met-Cy3glc
IL-6	1.15 (0.52)	0.80 (0.35)	0.71 (0.32)
iNOS	2.52 (0.79)	2.57 (1.15)	1.45 (0.52)
TNF-α	0.79 (0.05)*	0.78 (0.13)	0.71 (0.07)*
IL-1β	1.02 (0.11)	0.97 (0.16)	0.84 (0.14)
Ym-1	2.80 (1.2)	1.52 (0.87)	2.09 (1.03)
CCR2	ND	ND	ND
Arg-1	ND	ND	ND
CX3CR1	0.79 (0.42)	1.19 (0.53)	0.95 (0.47)

Results obtained were normalized for two housekeeping genes HPRT and cytochrome c1 by the 2-ΔΔCt method and expressed as mean (SEM). Statistical significance was assessed by one way ANOVA followed by Fisher's LSD multiple comparison test. *p<0.05

CCR2 and Arg1 expression was under detection levels and there were no significant differences in the expression of other tested markers.

3.2- Effect of cyanidin-3-glucose and derivatives under microglia LPS-stimulation

In order to induce a pro-inflammatory (M1) phenotype N9 microglia were stimulated with LPS. LPS markedly increase IL-6 and IL-1β expression, which as previously been associated with LPS stimulation (5). iNOS and TNF-α expression were not significantly changed by LPS induction (Table 3). The effect of the compounds was tested only on the M1 and M2 parameters significantly changed by LPS. Cy3glc treatment prevented LPS induced IL-6 increase. Curiously, Cy also prevented the IL-1β LPS-induced expression. CX3CR1 was not significantly affected by any of the tested conditions (Table 3).

3.3- Effect of cyanidin-3-glucose and derivatives on microglia under IL-4 stimulation

To understand if anthocyanins could potentiate the IL-4 induction to M2 phenotype, microglia cells were treated with IL-4 for 24 hours in the presence or absence of flavonoids. None of the tested parameters were significantly changed by IL-4 treatment. Stimulation with IL-4 curiously decreased CX3CR1, and this effect was independent of the presence of the compounds (Table 3).

Table 3- Effect of 1 µM of cyanidin (Cy), cyanidin-3-glucose (Cy3glc), 3' and 4'-Met-Cy3glc (Met-Cy3glc), on the transcription of microglia markers in induced N9 microglia cell line

	LPS				IL4			
	-	Cy	Cy3glc	Met-Cy3glc	-	Cy	Cy3glc	Met-Cy3glc
IL-6	81.85 (21.2)*	59.1 (12.47)*	37.50 (15.70)#	79.31 (15.04)*	0.66 (0.29)	-	-	-
iNOS	98.28 (52.24)	-	-	-	1.23 (0.09)	-	-	-
TNF-α	3.07 (0.93)	-	-	-	0.35 (0.15)	-	-	-
IL-1β	48.56 (15.13)*	40.69 (10.19)	55.43 (18.62)*	73.93 (20.81)*	0.40 (0.27)	-	-	-
Ym-1	ND	-	-	-	ND	-	-	-
CCR2	ND	-	-	-	ND	-	-	-
Arg-1	ND	-	-	-	ND	-	-	-
CX3CR1	0.78 (0.49)	0.65 (0.41)	2.85 (2.3)	1.10 (0.71)	0.15 (0.02)*	0.16 (0.02)*	0.20 (0.02)*	0.07 (0.03)*

Results obtained were normalized for two housekeeping genes HPRT and cytochrome c1 by the 2-ΔΔCt method and expressed as mean (SEM). Statistical significance was assessed by one way ANOVA followed by Fisher's LSD multiple comparison test. *p<0.05 vs control; #p<0.05 vs LPS

Table 4- Effect of microglia conditioned medium stimulated in the presence/absence of 1 µM of cyanidin (Cy), cyanidin-3-glucose (Cy3glc), 3' and 4'-Met-Cy3glc (Met-Cy3glc), on the neuronal transcription of CX3CL1 and synapsin.

SH-SY5Y	LPS				IL4			
	-	Cy	Cy3glc	Met-Cy3glc	-	Cy	Cy3glc	Met-Cy3glc
CX3CL1	1.15 (0.36)	2.30 (0.44)*#	0.76 (0.1)	0.93 (0.24)	1.28 (0.36)	1.23 (0.27)	0.79 (0.28)	1.23 (0.30)
Synapsin	3.81 (1.96)	3.32 (1.69)	2.74 (1.53)	1.11 (1.10)	1.04 (0.35)	0.77 (0.11)	1.02 (0.32)	0.91 (0.17)

Results obtained were normalized for two housekeeping genes HPRT and Eif4a2 by the 2-ΔΔCt method and expressed as mean (SEM). Statistical significance was assessed by one way ANOVA followed by Fisher's LSD multiple comparison test. *p<0.05 vs control; #p<0.05 vs LPS

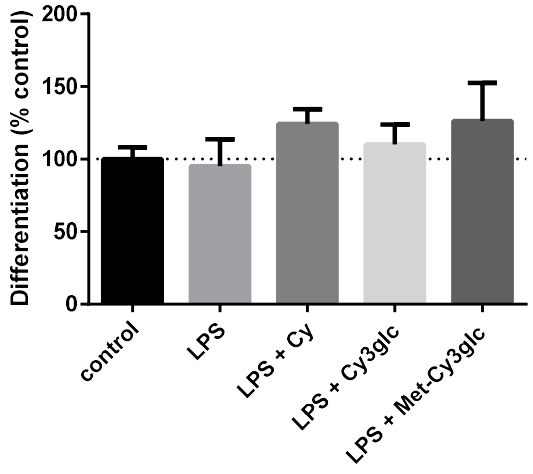


Figure 2- Effect of 1 µM of cyanidin (Cy), cyanidin-3-glucose (Cy3glc), 3' and 4'-Met-Cy3glc (Met-Cy3glc) on differentiation of neuronal cells SH-SY5Y. Cells bearing neurite-like processes longer than the diameter of the cell body were counted five randomly selected fields. The percentage of differentiation was calculated from the number of cells that showed process outgrowth divided by the total number of cells in each field. Results are expressed as percentage of control (mean ± SEM).

3.4- Neuronal response to conditioned medium of microglia stimulated with LPS or IL-4 in the presence/absence of flavonoids

The SH-SY5Y cell line was treated with the conditioned medium of the above conditions (Fig. 1B). As shown in table 4, LPS does not alter the CX3CL1 mRNA expression. Cyanidin did increase this expression comparing either with control and LPS. Results from neuronal differentiation may suggest an influence by Cy3glc and derivatives. (Fig. 2). CX3CL1 was not affected by IL-4 treatment either in the presence or absence of flavonoids (Table 4). Synapsin is a common neurotransmitter vesicular marker, used as an indicator of neurotransmission. In this study, synapsin expression was not significantly altered (table 4).

The direct effect of flavonoids in neuronal response to LPS activated conditioned microglia was also tested (Fig 1C). The results represented on Figure 3 confirmed the increase in fractalkine expression induced by cyanidin, simulating the effect obtained with the conditioned media (Table 4).

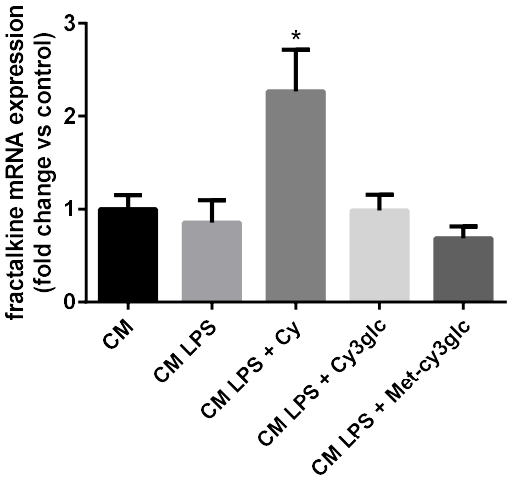


Figure 3 – Effect of 1 µM of cyanidin (Cy), cyanidin-3-glucose (Cy3glc), 3' and 4'-Met-Cy3glc (Met-Cy3glc) on the transcription of CX3CL1, in neuronal cells treated with conditioned medium (CM) from LPS-stimulated microglia. Results obtained were normalized for two housekeeping genes HPRT and Eif4a2 by the 2-ΔΔCt method and expressed as mean ± SEM. Statistical significance was assessed by one way ANOVA followed by Fisher's LSD multiple comparison test. *p<0.05 vs CM or CM LPS

4- Discussion

So far, the few studies using flavonoids tested them in order to examine changes in microglia "activation" (17). Nowadays it is known that besides failing to represent the non-pathological, normal functions of microglia, the term "activation" is vague and provides no specific information about the many possible microglial phenotypes (18). Previous work from our group has shown that in vivo supplementation with a blackberry anthocyanin extract induced some changes in synaptic related proteins (15). As microglia has been

implicated in synaptic pruning (24) it was hypothesized that flavonoids would have an impact in neuron function by changing microglia phenotype. Despite the inherent limitations of in vitro experiments, minimizing surrounding variables has the advantage of better understanding of direct causal effects (25). In this study the use of conditioned medium added a dynamic view of the overall process. Although not comprising physical interaction between the two cell types, this model allows the study of direct neuronal consequence of microglia released molecules, present on conditioned medium (23, 26). The retroviral immortalized microglia N9 cell line is generally considered a reliable in vitro model which enables the study of microglial function in the absence of contaminant cells which are present in primary cultures (27). Anthocyanins tested in this work were able to generally induce a less pro-inflammatory profile of N9 cells. Decreased TNF-α expression was induced either by cyanidin and methylated form of cyanidin-3-glucose.

These microglia cells were described as able to polarize in response to different stimulus (28). In the present study, LPS significantly increased IL-6 and IL-1β and seemed to increase iNOS and TNF-α. Liu et al 2012 (28) previously suggested that LPS induced polarization was irreversible. However, in this model, we tested the anthocyanins and LPS simultaneously expecting that this would hinder the changes induced by stimulation. This was supported by the decrease in IL-6 expression by Cy3glc. The anthocyanin on its native form was able to counteract the increase induced by LPS of IL-1β and also had a mild effect on IL-6 decrease. IL-1β is classically associated with inflammation, and LPS was able to induce an increase in IL-1β mRNA expression. Previous results of animal supplementation with blackberry extract rich in anthocyanins, has also shown an increase in IL-1β release simultaneously to increase in synaptic plasticity related molecules (15). Although classically associated with inflammatory profile, the role of this cytokine may not be completely unveiled since recent studies have suggested a role in neurogenesis and neuronal differentiation (29, 30).

Cy3glc and derivates counteracted LPS-induced inflammation to a lesser extent than shown to malvidin, another anthocyanidin, in a previous study (31). However the doses used in the present study were lower and probably more realistic. Anthocyanins were used at 1 μM, concentration that reflects reachable plasmatic concentrations (32). Not discarding the possibility that chronic consumption of anthocyanins rich-food can accumulate in tissues, as suggested by Kalt et colleagues 2008 (33), low μM or high nM are certainly the more reasonable concentrations to test on brain cells.

Healthy neurons constitutively express high levels of the chemokine CX3CL1 (also known as fractalkine), whereas the corresponding receptor CX3CR1 is found in microglia. The axis CX3CR1/CX3CL1 represents one of the most important axis between microglia and neurons (8). Impaired cognition was indeed observed in genetic knockout mice missing CX3CR1 (34). Contrary to what would be expected, the expression CX3CR1 on microglia cells was decreased by IL-4, either in the presence or absence of flavonoids, however absence of ligand regulation may be implicated. Despite flavonoids did not significantly increase this receptor, Cy did increase its ligand release by neurons. Cy3glc effect appeared to differ from other compounds tested, increasing CX3CR1 mRNA expression either in basal and LPS-stimulated cells. The subsequent application of flavonoids directly to neuronal cells allow to test a direct effect on prevention of microglia CM effects. Results confirmed the ability of the anthocyanidin to modulate neuronal fractalkine expression as already reported (15), even in a pro-inflammatory environment.

Selective modulation of microglia polarization to M2 phenotype has been presented as possible therapeutic target for some pathological conditions (35). Also, modulation of microglia biology affects neurons biology and plasticity, which can be helpful to increase its functionality even in physiological conditions (36). Microglia biology and its influence on neuronal function is a field that is evolving and concepts have been changing. This work results showed an attenuation of M1 phenotype. The M1/M2 polarization is not a standardized categorization, and although flavonoids are indeed able to decrease some pro-inflammatory cytokines as TNF-α, a marked effect on inducing alternative cytokines release was not seen. Although the usefulness of the terms M1 and M2, microglia cells are plastic and can shift between a spectrum of phenotypes instead of just fit in one closed classification. Anthocyanins and metabolites were not able to shift microglia to an M2 strict phenotype however they do interact with microglia biology, and furthermore with neuron biology, by increasing CX3CL1 expression. These results help to dig into the

current hot topic of microglia influence on neuronal biology and how flavonoids may be able to modulate this interaction.

Author Disclosure Statement

The authors declare no competing financial interests.

Aknowlegments

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References

1. Streit W, Mrak R, Griffin WS. Microglia and neuroinflammation: a pathological perspective. Journal of Neuroinflammation. 2004;1(1):14.
2. Morris GP, Clark IA, Zinn R, Vissel B. Microglia: a new frontier for synaptic plasticity, learning and memory, and neurodegenerative disease research. Neurobiol Learn Mem. 2013;105:40-53.
3. Wilcock DM. A changing perspective on the role of neuroinflammation in Alzheimer's disease. International journal of Alzheimer's disease. 2012;2012:495243.
4. Hu X, Leak RK, Shi Y, Suenaga J, Gao Y, Zheng P, et al. Microglial and macrophage polarization[mdash]new prospects for brain repair. Nat Rev Neurol. 2015;11(1):56-64.
5. Chhor V, Le Charpentier T, Lebon S, Ore MV, Celador IL, Josserand J, et al. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. Brain, behavior, and immunity. 2013;32:70-85.
6. Kim KH, Son SM, Mook-Jung I. Contributions of microglia to structural synaptic plasticity. J Exp Neurosci. 2013;7:85-91.
7. Tremblay ME, Stevens B, Sierra A, Wake H, Bessis A, Nimmerjahn A. The role of microglia in the healthy brain. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011;31(45):16064-9.
8. Limatola C, Ransohoff RM. Modulating neurotoxicity through CX3CL1/CX3CR1 signaling. Frontiers in Cellular Neuroscience. 2014;8.
9. Sheridan GK, Murphy KJ. Neuron-glia crosstalk in health and disease: fractalkine and CX3CR1 take centre stage. Open Biol. 2013;3(12):130181.
10. Letenneur L, Proust-Lima C, Le Gouge A, Dartigues JF, Barberger-Gateau P. Flavonoid intake and cognitive decline over a 10-year period. Am J Epidemiol. 2007;165(12):1364-71.
11. Kesse-Guyot E, Fezeu L, Andreeva VA, Touvier M, Scalbert A, Hercberg S, et al. Total and specific polyphenol intakes in midlife are associated with cognitive function measured 13 years later. J Nutr. 2012;142(1):76-83.
12. Spencer JP, Vafeiadou K, Williams RJ, Vauzour D. Neuroinflammation: modulation by flavonoids and mechanisms of action. Mol Aspects Med. 2012;33(1):83-97.
13. Meireles M, Martel F, Araujo J, Santos-Buelga C, Gonzalez-Manzano S, Duenas M, et al. Characterization and modulation of glucose uptake in a human blood-brain barrier model. J Membr Biol. 2013;246(9):669-77.
14. Martel F, Monteiro R, Calhau C. Effect of polyphenols on the intestinal and placental transport of some bioactive compounds. Nutr Res Rev. 2010;23(1):47-64.
15. Meireles M, Marques C, Norberto S, Fernandes I, Mateus N, Rendeiro C, et al. The impact of chronic blackberry intake on the neuroinflammatory status of rats fed a standard or high-fat diet. J Nutr Biochem. 2015.
16. Lau FC, Bielinski DF, Joseph JA. Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia. Journal of neuroscience research. 2007;85(5):1010-7.
17. Jang S, Johnson RW. Can consuming flavonoids restore old microglia to their youthful state? Nutrition reviews. 2010;68(12):719-28.
18. Cherry JD, Olschowka JA, O'Banion MK. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J Neuroinflammation. 2014;11:98.
19. Cherry JD, Olschowka JA, O'Banion MK. Are "resting" microglia more "m2"? Front Immunol. 2014;5:594.
20. Penna I, Vella S, Gigoni A, Russo C, Cancedda R, Pagano A. Selection of candidate housekeeping genes for normalization in human postmortem brain samples. Int J Mol Sci. 2011;12(9):5461-70.
21. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8.
22. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):RESEARCH0034.
23. Munch G, Gasic-Milenkovic J, Dukic-Stefanovic S, Kuhla B, Heinrich K, Riederer P, et al. Microglial activation induces cell death, inhibits neurite outgrowth and causes neurite retraction of differentiated neuroblastoma cells. Experimental brain research. 2003;150(1):1-8.
24. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, et al. Synaptic pruning by microglia is necessary for normal brain development. Science. 2011;333(6048):1456-8.
25. Carson MJ, Crane J, Xie AX. Modeling CNS microglia: the quest to identify predictive models. Drug discovery today Disease models. 2008;5(1):19-25.
26. Wenker SD, Chamorro ME, Vittori DC, Nesse AB. Protective action of erythropoietin on neuronal

damage induced by activated microglia. The FEBS journal. 2013;280(7):1630-42.
27. Stansley B, Post J, Hensley K. A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. J Neuroinflammation. 2012;9:115.
28. Liu HC, Zheng MH, Du YL, Wang L, Kuang F, Qin HY, et al. N9 microglial cells polarized by LPS and IL4 show differential responses to secondary environmental stimuli. Cell Immunol. 2012;278(1-2):84-90.
29. Xue F, Liu L, Fan J, He S, Li R, Peng ZW, et al. Interleukin-1beta promotes the neurogenesis of carotid bodies by stimulating the activation of ERK1/2. Respiratory physiology & neurobiology. 2015;219:78-84.
30. Seguin JA, Brennan J, Mangano E, Hayley S. Proinflammatory cytokines differentially influence adult hippocampal cell proliferation depending upon the route and chronicity of administration. Neuropsychiatric Disease and Treatment. 2009;5:5-14.
31. Bognar E, Sarszegi Z, Szabo A, Debreceni B, Kalman N, Tucsek Z, et al. Antioxidant and anti-inflammatory effects in RAW264.7 macrophages of malvidin, a major red wine polyphenol. PLoS One. 2013;8(6):e65355.
32. Czank C, Cassidy A, Zhang Q, Morrison DJ, Preston T, Kroon PA, et al. Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a (13)C-tracer study. Am J Clin Nutr. 2013;97(5):995-1003.
33. Kalt W, Blumberg JB, McDonald JE, Vinqvist-Tymchuk MR, Fillmore SA, Graf BA, et al. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. Journal of agricultural and food chemistry. 2008;56(3):705-12.
34. Rogers JT, Morganti JM, Bachstetter AD, Hudson CE, Peters MM, Grimmig BA, et al. CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011;31(45):16241-50.
35. Xia C-Y, Zhang S, Gao Y, Wang Z-Z, Chen N-H. Selective modulation of microglia polarization to M2 phenotype for stroke treatment. International Immunopharmacology. 2015;25(2):377-82.
36. Hu X, Leak RK, Shi Y, Suenaga J, Gao Y, Zheng P, et al. Microglial and macrophage polarization-new prospects for brain repair. Nat Rev Neurol. 2015;11(1):56-64.

CHAPTER VII

Concluding remarks

With the increase in life expectancy, chronic diseases that affect brain function have also increased. Nowadays people are currently exposed to demanding tasks and keeping a healthy brain, either to optimizing its function or delaying its complications typical of ageing, is crucial. Flavonoids, which have been extensively studied for their potential to treat cardiovascular diseases and cancer, have been attracting the interest of scientific community for its neuroprotective potential. The compilation of works presented in this thesis aimed to expand the understanding of the effects of flavonoids on brain function and to explore its therapeutic potential.

A strong body of literature has shown the ability of flavonoids and other polyphenols to modulate several membrane transports at intestinal and placental barrier (Martel, Monteiro et al. 2010). Blood-brain barrier (BBB) is one of the tightest barriers in mammalians that regulate the access of a plurality of bioactive compounds to the brain. Ability of flavonoids to cross this barrier has already been shown (Youdim, Dobbie et al. 2003, Faria, Pestana et al. 2010, Faria, Meireles et al. 2014), however there was lack of knowledge about the effects they can have on membrane transport of other compounds at this level.

Glucose is the main fuel to brain cells, which even in rest are able to consume 20% of energy necessary to meet our basal demands. To work properly, our brain needs, at first instance, energy. Paradoxically, brain has limited stores of glycogen (Owen and Sunram-Lea 2011) and it depends on a tight regulation of glucose to maintain its homeostasis. BBB can adapt the glucose transport in response to neuroglial signals to adjust to brain demands (Leybaert, De Bock et al. 2007). However, this process is not always perfect - poor glucose regulation occurs and is associated with memory impairment and age-related cognitive deficits. Furthermore, previous

research has demonstrated that glucose administration can enhance learning and memory in healthy young and aged animals and humans and may even improve several cognitive functions in individuals with Alzheimer's disease (Craft, Zallen et al. 1992, Messier, Gagnon et al. 1997) and Down's syndrome (Manning, Honn et al. 1998) whose cognition is severely compromised. Furthermore, impairments in certain cognitive skills have been recognized as a possible complication of diabetes mellitus, and evidence exists that improved glycemic control can ameliorate performance on selective areas of cognition in these populations (Seto, Yang et al. 2015). Glucose administration as enhancer of memory performance in healthy young adults has also been suggested (Sunram-Lea, Foster et al. 2001).

In the present work we verified that flavonoids are important modulators of glucose levels also at BBB level in addition to modulation at other biological barriers as previously mentioned. Curiously, results showed that flavonoids belonging to two different structural classes have different effects on glucose modulation. The flavan-3-ols catechin and epicatechin did not have any effect on [^{3}H]-DG uptake. However their methylated forms were able to increase this uptake. The effect with flavonols was in a different direction: both quercetin and miricetin decreased glucose uptake in a concentration-dependent manner. However, the glucuronic acid conjugated of quercetin, one of the most abundant circulating metabolites of quercetin after ingestion of a quercetin-rich meal (Mullen, Boitier et al. 2004), produced no effect on glucose uptake in BBB which could support a reduced impact of quercetin ingestion on glucose uptake. Indeed, metabolization can affect flavonoids action, which can be an indirect way for the organism to control and modify flavonoids action and restore homeostasis (Williamson 2013).

Some of the questions that could be raised after the results of the chapter II is whether glucose facilitation can have beneficial or detrimental effects. Taking in account that glucose effects are U inverted shaped, the answer for this question would depend on the requirement of brain for glucose, which is known to change according to the tasks demanded to the brain (van der Zwaluw, van de Rest et al. 2015).

Once brain energetic needs are accomplished, neurological functions are tightly dependent on the neurotransmitters released. Some of the most common neurological diseases are directly or indirectly related to problems with neurotransmitters. These neurotransmitters as well as other endogenous bioactive amines and vitamins are organic cations and depend on organic cation transporters systems (Koepsell, Lips et al. 2007).

In chapter III we sought to explore whether flavonoids were able important transport systems also at neuronal level and by these way account for its neuroprotection potential. [^{3}H]-1-Methyl-4-phenylpyridinium ([^{3}H]-MPP$^+$) is a classic substrate for organic cation transporters and previous studies had already shown that MPP$^+$ uptake was susceptible to flavonoids interference at intestinal levels. However, at neuronal level, the transporters involved are different and there were no information about flavonoids action there. SH-SY5Y cell line is commonly used as model of dopaminergic neurons. After differentiation these cells acquire a dopaminergic phenotype, characterized by expression of enzyme tyrosine hydroxylase and dopamine transporter (DAT) (Presgraves, Ahmed et al. 2004). The DAT is responsible for recycling dopamine (DA) from the synaptic cleft and modulation at this level would for instance regulate levels of dopamine available for neurotransmission. Depression, bipolar disorder, Parkinson's disease, and attention deficit hyperactivity disorder are associated with abnormal dopamine levels and DAT may be implicated in some of these conditions. Inhibitors or agonists of DAT are being considered as new approaches to treat these disorders (Vaughan and Foster 2013).

The results presented in this chapter showed a partial involvement of DAT on [^{3}H]-MPP$^+$, uptake, and therefore, a possible involvement of flavonoids on modulation of this transporter. Inhibition of [^{3}H]-MPP$^+$ uptake by flavan-3-ols can affect neurotransmission by increasing dopamine on the synaptic cleft. However, naringenin, hesperitin, quercetin and metabolites increased this uptake. This increase can be helpful in psychoses where synaptic cleft levels of

dopamine are increased as schizophrenia or situations of drugs abuse like methamphetamines and cocaine (Sekine, Iyo et al. 2001, Deik, Saunders-Pullman et al. 2012).

Overall, this work showed that organic cations uptake in dopaminergic neurons may be modulated by diet flavonoids and most interesting, by its biological metabolites, in structural-dependent way. This uptake seemed to be regulated by Ca^{2+} pathways, phosphorylation/dephosphorylation mechanisms and have a partial involvement of DAT. Interference of flavonoids with multiple signaling cascades has been review by Spencer et al., 2009 (Spencer 2009). This include selective actions on a number of protein kinases and lipid kinase signaling cascades, most notably the PI3K/Akt and MAP kinase pathways which regulate pro-survival transcription factors and gene expression. Inhibition of apoptosis triggered by neurotoxic species represents a way to maintain a good number of functional neurons. Notwithstanding, the potential to inhibit or stimulate important transport systems may change the communication between these cells which may be relevant to explain the potential neuroprotective actions of some flavonoids.

Chapter II shows flavonoids' ability to modulate glucose uptake at BBB which may explain some of beneficial actions previously seen with flavonoids. Although glucose is necessary for adequately brain function, an excess on plasmatic glucose levels as seen in diabetic patients is an established risk factor for cognitive impairment. On chapter III it was observed an ability of flavonoids to modulate organic cation uptake which may have impact on neurotransmitters availability and synapse function. The connection between these two approaches was made on chapter IV where was tested the long-term consumption effects of flavonoids in the dopaminergic system, in diet-induced obese rats, where hyperglycaemia was expected.

There is a widespread concern regarding the effects of modern, typically westernized dietary patterns, characterized by high-fat and high-sugar foods. These diets have been mimicked in rats and used as model to induce obesity and the co-morbidities associated. *In vivo* studies have shown that high-fat diet fed animals have several brain disorders among them, dysfunction of

dopaminergic system and of neurogenesis impairment (Kanoski and Davidson 2011). Berries, natural source of flavonoids, have attracted interest as nutritional approach to improve health, metabolic and cognitive outcomes (Basu, Rhone et al. 2010, Rendeiro, Guerreiro et al. 2012, Norberto, Silva et al. 2013). Extract from blackberries was chosen due its natural high content on anthocyanins, characterized mainly by cyanidin-3-glucose. Also, previous studies had pointed blackberry extracts as improvers of cognitive function and antioxidant status in animal studies (Shukitt-Hale, Cheng et al. 2009, Hassimotto and Lajolo 2011).

Previous studies have suggested an association between glucose and dopamine levels, with low levels of glucose in the brain inhibiting DA release. Animal supplementation with blackberry extract (BE) decreased dopamine content in striatum and increased DOPAC/DA ratio indicating a higher DA turnover with BE supplementation. Also, BE supplementation acted towards an insulin mimetic direction reinforcing the involvement with glucose systems. This study also confirmed that high-fat, high-carbohydrate diet induced obesity can prompt several features of metabolic dysfunction in Wistar rats, some of them being partially reverted with low doses of blackberry extract.

Taking into account that neuroinflammation has been suggested as a central mediator of central nervous system (CNS) dysfunction (Yirmiya, Rimmerman et al. 2015), the following work (described in chapter V) aimed to assess the impact of a chronic blackberry anthocyanin extract supplementation on the neuroinflammatory profile using the *in vivo* design described on chapter IV. Results obtained in this study went further than showing the potential of anthocyanins to counteract diet-induced inflammation, but also suggested that, without an inflammatory context, anthocyanins are able to modulate processes involved in synaptic plasticity. PDGF-AA and fractalkine, as example, were consistently modulated by BE supplementation in the cortex and the hippocampus of standard fed animals. Moreover, the BE supplementation also increased cortex expression of activin A, agrin, and VEGF, all known to be important in synapse structure, regeneration and synaptogenesis (Shoji-Kasai, Ageta et al. 2007, Rosenstein, Krum et

al. 2010, Daniels 2012). The modulation of fractalkine expression by BE was one of the most consistent findings, since it was increased in both cortex and hippocampus of supplemented animals, independently of fat diet content. Fractalkine is a chemokine synthetized by neurons, whose receptor is highly expressed in microglia cells (Kettenmann, Kirchhoff et al. 2013). Fractalkine (CX3CL1) and its receptor (CX3CR1) represent an important axis in neuronal/microglial crosstalk and modulation at this axis may be essential in maintaining microglia in a resting state in the healthy brain as well as preventing excessive microglial activation during inflammatory conditions (Jurgens and Johnson 2012, Wolf, Yona et al. 2013). Previous data suggested that flavonoids were capable of changing the phenotype of microglia by restoring the population of microglial cells to the "resting" state from the more active state (Jang and Johnson 2010).

To further explore the ability of BE to modulate fractalkine expression, anthocyanins and their physiological metabolites were assessed *in vitro* for their potential to influence neuronal fractalkine mRNA expression. Cyanidin markedly increased the expression of fractalkine at the concentration of 1 µM, whilst cyanidin-3-glucoside and metabolites induced a significant reduction on fractalkine mRNA levels. Despite the disparity between our *in vitro* and *in vivo* models, the *in vitro* data confirm the potential of flavonoid aglycones to affect fractalkine expression. Nevertheless, *in vitro* models lack the complexity of the CNS, notably here the absence of microglia and astrocytes which may be required for the optimal regulation of inflammatory proteins. The following work, described in chapter VI, took into account the dynamic microglia-neuron using conditioned medium of treated or untreated microglia to culture neurons for 24 hours.

From the results of chapter V and the continuous literature being published about microglia phenotype and the new roles of microglia in brain, a new hypothesis was raised: are flavonoids able to change synaptic plasticity? And if so, are this changes dependent on microglia phenotype? Results from these work pointed the effects of synaptic plasticity as not so depend

on microglia phenotype as hypothesized. Anthocyanins and metabolites were not able to shift microglia to an M2 strict phenotype however they did interact with microglia biology, showing an attenuation of M1 phenotype, and furthermore with neuron biology, increasing CX3XL1 expression.

The works presented in chapter V and VI suggested an influence of flavonoids, specifically anthocyanins, in the stimulation of transcription and production of molecules involved in synaptic plasticity. One of the most remarkable capabilities of the brain it is its ability to respond and adapt accordingly to different stimulus. This brain plasticity is characterized by the ability to increase number of mature neurons from neural stem cells – neurogenesis – and by changing the connections between the existent ones – synaptic plasticity. Synaptic plasticity is closely associated to microglia function which has a determinant role in synaptic pruning. Diet is among the environmental factors that stimulates and impacts brain function either by changing synaptic plasticity or microglia function (Stangl and Thuret 2009, Johnson 2015) and here anthocyanins showed some impact on these processes either *in vivo* and *in vitro* models.

Flavonoids and other polyphenols in general keep on the top list of promising dietary interventions to impact brain function along with caloric restriction, intermittent fasting, and poly-unsaturated fatty acids (PUFA) (Murphy, Dias et al. 2014). Caloric restriction has been extensively studied due to its “healthspan” potential including delaying the onset of age-related process, as cognitive decline. It appears to improve the resistance of synapses to metabolic and oxidative damage. Also the total number, structure, and functional status of synapses and induces the production of neurotrophic factors can be modulated by caloric restriction (Mattson 2012). Dietary interventions that could mimic the effects of caloric restriction without the constraint of food intake has become an attractive field of research. On this regard, resveratrol, naturally occurring in grapes and wine, has attracted scientific community interest (Baur 2010). It could be interesting to establish if the structural close flavonoids are able to prevent cognitive decline also by acting on the same molecular targets as caloric restriction.

The future in flavonoid research will work on establishing a dietary recommendation intake for flavonoids and other bioactive compounds as has been recently discussed by a panel of experts. Although the challenge, establishing a reference intake value would help scientists to design convergent studies and lead science to a faster progress (Lupton, Atkinson et al. 2014). The habitual intake of flavonoids in Europe is below the amounts found to have a significant health effect (Vogiatzoglou, Mulligan et al. 2015). It would be beneficial for consumers to set a goal for the amount needed to incorporate in diet (Williamson and Holst 2008). This is only possible after well designed randomized controlled trials with dose-efficacy and safety tests. Short-term clinical trials could focus on cognitive disorders whereas long-term trials could focus on prevention and treatment of neurodegenerative diseases and age-related brain dysfunction (Lupton, Atkinson et al. 2014, Balentine, Dwyer et al. 2015). A few years ago clinical trials on flavonoids and cognition were sparse (Macready, Kennedy et al. 2009). At the present several trials on cognition are ongoing and this knowledge will be continuously increased.

The work presented in this thesis pointed flavonoids as modulators of metabolism, either in physiological conditions or by changing the response induced by high-fat diet (Figure 2). Modulation of glucose metabolism had a special relevance since flavonoids changed the access of glucose by CNS as well as presented insulin mimetic actions *in vivo*. Flavonoids had also impact on CNS parameters that may be directed or undirected related with metabolism. Flavonoids were able to modulate dopaminergic system, modify markers of classic activated microglia and counteract neuroinflammation induced by high-fat diet *in vivo.* Modulation of transporters and proteins involved in synaptic plasticity by flavonoids may be also relevant to restoring homeostasis in pathological conditions or could represent an improvement in physiological brain functions.

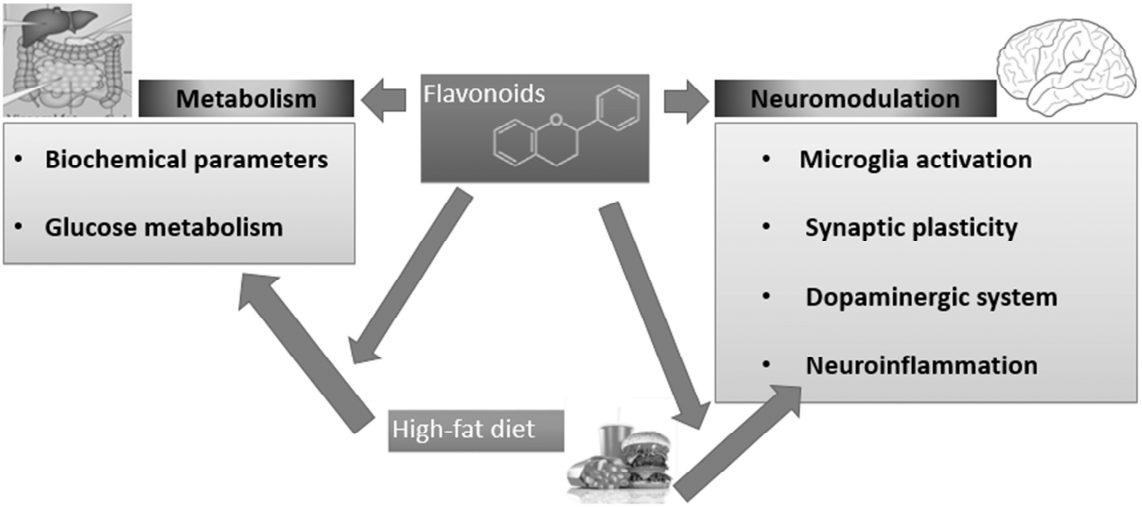

Figure 2-Schematic representation of the effects of flavonoids explored in this work.

It was also clear that structural difference between flavonoids, natural or as result of metabolization can impact their biological activity. Chronic supplementation with an anthocyanin rich extract allowed us to explore some of the mechanisms proposed *in vitro* and to suggest new ones. This work presented some explanations which may help to determine and understand the biological efficacy of flavonoids. Moreover, the effects of flavonoids explored here can give important clues about which specific outcomes to measure and explore in future clinical trials. Given the high prevalence of dementia, even a small impact on delaying cognitive decline might have a serious impact on public health.

REFERENCES

Abbott, N. J., A. A. Patabendige, D. E. Dolman, S. R. Yusof and D. J. Begley (2010). "Structure and function of the blood-brain barrier." Neurobiol Dis 37(1): 13-25.

Abd El Mohsen, M. M., G. Kuhnle, A. R. Rechner, H. Schroeter, S. Rose, P. Jenner and C. A. Rice-Evans (2002). "Uptake and metabolism of epicatechin and its access to the brain after oral ingestion." Free Radic Biol and Med 33(12): 1693-1702.

Aherne, S. A. and N. M. O'Brien (2002). "Dietary flavonols: chemistry, food content, and metabolism." Nutrition 18(1): 75-81.

Balentine, D. A., J. T. Dwyer, J. W. Erdman, Jr., M. G. Ferruzzi, P. C. Gaine, J. M. Harnly and C. L. Kwik-Uribe (2015). "Recommendations on reporting requirements for flavonoids in research." Am J Clin Nutr 101(6): 1113-1125.

Basu, A., M. Rhone and T. J. Lyons (2010). "Berries: emerging impact on cardiovascular health." Nutr Rev 68(3): 168-177.

Baur, J. A. (2010). "Resveratrol, sirtuins, and the promise of a DR mimetic." Mech Ageing Dev 131(4): 261-269.

Beecher, G. R. (2003). "Overview of dietary flavonoids: nomenclature, occurrence and intake." J Nutr 133(10): 3248S-3254S.

Bors, W., W. Heller, C. Michel and M. Saran (1990). [36] Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. Methods in Enzymology, Academic Press. Volume 186: 343-355.

Cassidy, A., K. J. Mukamal, L. Liu, M. Franz, A. H. Eliassen and E. B. Rimm (2013). "High Anthocyanin Intake Is Associated With a Reduced Risk of Myocardial Infarction in Young and Middle-Aged Women." Circulation 127(2): 188-196.

Chen, T.-Y., J. Kritchevsky, K. Hargett, K. Feller, R. Klobusnik, B. J. Song, B. Cooper, Z. Jouni, M. G. Ferruzzi and E. M. Janle (2015). "Plasma bioavailability and regional brain distribution of polyphenols from apple/grape seed and bilberry extracts in a young swine model." Molecular Nutrition & Food Research: n/a-n/a.

Commenges, D., V. Scotet, S. Renaud, H. Jacqmin-Gadda, P. Barberger-Gateau and J. F. Dartigues (2000). "Intake of flavonoids and risk of dementia." Eur J Epidemiol 16(4): 357-363.

Corcoran, M. P., D. L. McKay and J. B. Blumberg (2012). "Flavonoid basics: chemistry, sources, mechanisms of action, and safety." J Nutr Gerontol Geriatr 31(3): 176-189.

Craft, S., G. Zallen and L. D. Baker (1992). "Glucose and memory in mild senile dementia of the Alzheimer type." J Clin Exp Neuropsychol 14(2): 253-267.

Daniels, M. P. (2012). "The role of agrin in synaptic development, plasticity and signaling in the central nervous system." Neurochem Int 61(6): 848-853.

Day, A. J., F. J. Canada, J. C. Diaz, P. A. Kroon, R. McLauchlan, C. B. Faulds, G. W. Plumb, M. R. Morgan and G. Williamson (2000). "Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase." FEBS Lett 468(2-3): 166-170.

Deik, A., R. Saunders-Pullman and M. S. Luciano (2012). "Substance abuse and movement disorders: complex interactions and comorbidities." Curr Drug Abuse Rev 5(3): 243-253.

Egert, S. and G. Rimbach (2011). "Which sources of flavonoids: complex diets or dietary supplements?" Adv Nutr 2(1): 8-14.

El Mohsen, M. A., J. Marks, G. Kuhnle, K. Moore, E. Debnam, S. K. Srai, C. Rice-Evans and J. P. E. Spencer (2006). "Absorption, tissue distribution and excretion of pelargonidin and its metabolites following oral administration to rats." Br J of Nut 95(01): 51-58.

Engelhart, M. J., M. I. Geerlings, A. Ruitenberg, J. C. van Swieten, A. Hofman, J. C. Witteman and M. M. Breteler (2002). "Dietary intake of antioxidants and risk of Alzheimer disease." Jama 287(24): 3223-3229.

Faria, A., I. Fernandes, S. Norberto, N. Mateus and C. Calhau (2014). "Interplay between anthocyanins and gut microbiota." J Agric Food Chem 62(29): 6898-6902.

Faria, A., M. Meireles, I. Fernandes, C. Santos-Buelga, S. Gonzalez-Manzano, M. Duenas, V. de Freitas, N. Mateus and C. Calhau (2014). "Flavonoid metabolites transport across a human BBB model." Food Chem 149: 190-196.

Faria, A., D. Pestana, J. Azevedo, F. Martel, V. de Freitas, I. Azevedo, N. Mateus and C. Calhau (2009). "Absorption of anthocyanins through intestinal epithelial cells - Putative involvement of GLUT2." Mol Nutr Food Res 53(11): 1430-1437.

Faria, A., D. Pestana, D. Teixeira, J. Azevedo, V. De Freitas, N. Mateus and C. Calhau (2010). "Flavonoid transport across RBE4 cells: A blood-brain barrier model." Cell Mol Biol Lett 15(2): 234-241.

Ferruzzi, M. G., J. K. Lobo, E. M. Janle, B. Cooper, J. E. Simon, Q. L. Wu, C. Welch, L. Ho, C. Weaver and G. M. Pasinetti (2009). "Bioavailability of gallic acid and catechins from grape seed

polyphenol extract is improved by repeated dosing in rats: implications for treatment in Alzheimer's disease." J Alzheimers Dis 18(1): 113-124.

Foster, J. A. and K.-A. McVey Neufeld (2013). "Gut–brain axis: how the microbiome influences anxiety and depression." Trends in Neurosci 36(5): 305-312.

Gao, X., A. Cassidy, M. A. Schwarzschild, E. B. Rimm and A. Ascherio (2012). "Habitual intake of dietary flavonoids and risk of Parkinson disease." Neurology 78(15): 1138-1145.

Gorelick, P. B. (2010). "Role of inflammation in cognitive impairment: results of observational epidemiological studies and clinical trials." Ann N Y Acad Sci 1207: 155-162.

Hassimotto, N. M. and F. M. Lajolo (2011). "Antioxidant status in rats after long-term intake of anthocyanins and ellagitannins from blackberries." J Sci Food Agric 91(3): 523-531.

Heim, K. E., A. R. Tagliaferro and D. J. Bobilya (2002). "Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships." J Nutr Biochem 13(10): 572-584.

Hirsch, E. C. and S. Hunot (2009). "Neuroinflammation in Parkinson's disease: a target for neuroprotection?" Lancet Neurol 8(4): 382-397.

Hooper, L., P. A. Kroon, E. B. Rimm, J. S. Cohn, I. Harvey, K. A. Le Cornu, J. J. Ryder, W. L. Hall and A. Cassidy (2008). "Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials." Am J Clin Nutr 88(1): 38-50.

Jang, S. and R. W. Johnson (2010). "Can consuming flavonoids restore old microglia to their youthful state?" Nutr Rev 68(12): 719-728.

Jennings, A., A. A. Welch, S. J. Fairweather-Tait, C. Kay, A.-M. Minihane, P. Chowienczyk, B. Jiang, M. Cecelja, T. Spector, A. Macgregor and A. Cassidy (2012). "Higher anthocyanin intake is associated with lower arterial stiffness and central blood pressure in women." Am J Clin Nut 96(4): 781-788.

Johnson, R. W. (2015). "Feeding the beast: can microglia in the senescent brain be regulated by diet?" Brain Behav Immun 43: 1-8.

Joseph, J. A., B. Shukitt-Hale and L. M. Willis (2009). "Grape juice, berries, and walnuts affect brain aging and behavior." J Nutr 139(9): 1813S-1817S.

Jurgens, H. A. and R. W. Johnson (2012). "Dysregulated neuronal-microglial cross-talk during aging, stress and inflammation." Exp Neurol 233(1): 40-48.

Kanoski, S. E. and T. L. Davidson (2011). "Western diet consumption and cognitive impairment: links to hippocampal dysfunction and obesity." Physiol Behav 103(1): 59-68.

Kean, R. J., D. J. Lamport, G. F. Dodd, J. E. Freeman, C. M. Williams, J. A. Ellis, L. T. Butler and J. P. Spencer (2015). "Chronic consumption of flavanone-rich orange juice is associated with cognitive benefits: an 8-wk, randomized, double-blind, placebo-controlled trial in healthy older adults." Am J Clin Nutr 101(3): 506-514.

Kennedy, D. O. and E. L. Wightman (2011). "Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function." Adv Nutr 2(1): 32-50.

Kesse-Guyot, E., L. Fezeu, V. A. Andreeva, M. Touvier, A. Scalbert, S. Hercberg and P. Galan (2012). "Total and specific polyphenol intakes in midlife are associated with cognitive function measured 13 years later." J Nutr 142(1): 76-83.

Kettenmann, H., F. Kirchhoff and A. Verkhratsky (2013). "Microglia: new roles for the synaptic stripper." Neuron 77(1): 10-18.

Koepsell, H., K. Lips and C. Volk (2007). "Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications." Pharm Res 24(7): 1227-1251.

Krikorian, R., T. A. Nash, M. D. Shidler, B. Shukitt-Hale and J. A. Joseph (2010). "Concord grape juice supplementation improves memory function in older adults with mild cognitive impairment." Br J Nutr 103(5): 730-734.

Krikorian, R., M. D. Shidler, T. A. Nash, W. Kalt, M. R. Vinqvist-Tymchuk, B. Shukitt-Hale and J. A. Joseph (2010). "Blueberry supplementation improves memory in older adults." J Agric Food Chem 58(7): 3996-4000.

Letenneur, L., C. Proust-Lima, A. Le Gouge, J. F. Dartigues and P. Barberger-Gateau (2007). "Flavonoid intake and cognitive decline over a 10-year period." Am J Epidemiol 165(12): 1364-1371.

Leybaert, L., M. De Bock, M. Van Moorhem, E. Decrock and E. De Vuyst (2007). "Neurobarrier coupling in the brain: adjusting glucose entry with demand." J Neurosci Res 85(15): 3213-3220.

Lieberman, H. R. (2001). "The effects of ginseng, ephedrine, and caffeine on cognitive performance, mood and energy." Nutr Rev 59(4): 91-102.

Lin, S. P., Y. C. Hou, S. Y. Tsai, M. J. Wang and P. D. Chao (2014). "Tissue distribution of naringenin conjugated metabolites following repeated dosing of naringin to rats." Biomedicine 4(16): 13.

Loef, M. and H. Walach (2012). "Fruit, vegetables and prevention of cognitive decline or dementia: a systematic review of cohort studies." J Nutr Health Aging 16(7): 626-630.

Lupton, J. R., S. A. Atkinson, N. Chang, C. G. Fraga, J. Levy, M. Messina, D. P. Richardson, B. van Ommen, Y. Yang, J. C. Griffiths and J. Hathcock (2014). "Exploring the benefits and challenges of establishing a DRI-like process for bioactives." Eur J Nutr 53 Suppl 1: 1-9.

Macready, A. L., O. B. Kennedy, J. A. Ellis, C. M. Williams, J. P. Spencer and L. T. Butler (2009). "Flavonoids and cognitive function: a review of human randomized controlled trial studies and recommendations for future studies." Genes Nutr 4(4): 227-242.

Manach, C., A. Scalbert, C. Morand, C. Remesy and L. Jimenez (2004). "Polyphenols: food sources and bioavailability." Am J Clin Nutr 79(5): 727-747.

Manach, C., G. Williamson, C. Morand, A. Scalbert and C. Remesy (2005). "Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies." Am J Clin Nutr 81(1 Suppl): 230S-242S.

Manning, C. A., V. J. Honn, W. S. Stone, J. S. Jane and P. E. Gold (1998). "Glucose effects on cognition in adults with Down's syndrome." Neuropsychology 12(3): 479-484.

Martel, F., R. Monteiro and C. Calhau (2010). "Effect of polyphenols on the intestinal and placental transport of some bioactive compounds." Nutr Res Rev 23(1): 47-64.

Maslen, H., N. Faulmuller and J. Savulescu (2014). "Pharmacological cognitive enhancement-how neuroscientific research could advance ethical debate." Front Syst Neurosci 8(107).

Mattson, M. P. (2012). "Energy intake and exercise as determinants of brain health and vulnerability to injury and disease." Cell Metab 16(6): 706-722.

Messier, C., M. Gagnon and V. Knott (1997). "Effect of glucose and peripheral glucose regulation on memory in the elderly." Neurobiol Aging 18(3): 297-304.

Mullen, W., A. Boitier, A. J. Stewart and A. Crozier (2004). "Flavonoid metabolites in human plasma and urine after the consumption of red onions: analysis by liquid chromatography with photodiode array and full scan tandem mass spectrometric detection." J Chromatogr A 1058(1-2): 163-168.

Muralidhara, K. G. (2015). Chapter 29 - Dietary Supplements as Cognitive Enhancers: The Role of Flavonoid-Rich Foods and their Relevance in Age-Related Neurodegeneration. Bioactive Nutraceuticals and Dietary Supplements in Neurological and Brain Disease. R. R. W. R. Preedy. San Diego, Academic Press: 281-290.

Murphy, T., G. P. Dias and S. Thuret (2014). "Effects of diet on brain plasticity in animal and human studies: mind the gap." Neural Plast 2014: 563160.

Negrão, R. and A. Faria (2009). Natural Polyphenols as Anti-Oxidant, Anti-Inflammatory and Anti-Angiogenic Agents in the Metabolic Syndrome. Oxidative Stress, Inflammation and Angiogenesis in the Metabolic Syndrome. R. Soares and C. Costa, Springer Netherlands: 147-180.

Nemeth, K., G. W. Plumb, J. G. Berrin, N. Juge, R. Jacob, H. Y. Naim, G. Williamson, D. M. Swallow and P. A. Kroon (2003). "Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans." Eur J Nutr 42(1): 29-42.

Norberto, S., S. Silva, M. Meireles, A. Faria, M. Pintado and C. Calhau (2013). "Blueberry anthocyanins in health promotion: A metabolic overview." J Funct Foods 5(4): 1518-1528.

Nurmi, T., J. Mursu, M. Heinonen, A. Nurmi, R. Hiltunen and S. Voutilainen (2009). "Metabolism of Berry Anthocyanins to Phenolic Acids in Humans." J Agric Food Chem 57(6): 2274-2281.

Owen, L. and S. I. Sunram-Lea (2011). "Metabolic agents that enhance ATP can improve cognitive functioning: a review of the evidence for glucose, oxygen, pyruvate, creatine, and L-carnitine." Nutrients 3(8): 735-755.

Palmer, A. M. (2010). "The blood-brain barrier." Neurobiology of Disease 37(1): 1-2.

Presgraves, S. P., T. Ahmed, S. Borwege and J. N. Joyce (2004). "Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists." Neurotox Res 5(8): 579-598.

Rendeiro, C., J. D. Guerreiro, C. M. Williams and J. P. Spencer (2012). "Flavonoids as modulators of memory and learning: molecular interactions resulting in behavioural effects." Proc Nutr Soc 71(2): 246-262.

Rendeiro, C., D. Vauzour, R. J. Kean, L. T. Butler, M. Rattray, J. P. Spencer and C. M. Williams (2012). "Blueberry supplementation induces spatial memory improvements and region-specific regulation of hippocampal BDNF mRNA expression in young rats." Psychopharmacology (Berl) 223(3): 319-330.

Rice-Evans, C. A., N. J. Miller and G. Paganga (1996). "Structure-antioxidant activity relationships of flavonoids and phenolic acids." Free Radic Biol Med 20(7): 933-956.

Rodrigues, M. C., P. R. Sanberg, L. E. Cruz and S. Garbuzova-Davis (2014). "The innate and adaptive immunological aspects in neurodegenerative diseases." J Neuroimmunol 269(1-2): 1-8.

Rosenstein, J. M., J. M. Krum and C. Ruhrberg (2010). "VEGF in the nervous system." Organogenesis 6(2): 107-114.

Scalbert, A. and G. Williamson (2000). "Dietary intake and bioavailability of polyphenols." J Nutr 130(8S Suppl): 2073S-2085S.

Schaffer, S., H. Asseburg, S. Kuntz, W. E. Muller and G. P. Eckert (2012). "Effects of polyphenols on brain ageing and Alzheimer's disease: focus on mitochondria." Mol Neurobiol 46(1): 161-178.

Sekine, Y., M. Iyo, Y. Ouchi, T. Matsunaga, H. Tsukada, H. Okada, E. Yoshikawa, M. Futatsubashi, N. Takei and N. Mori (2001). "Methamphetamine-related psychiatric symptoms and reduced brain dopamine transporters studied with PET." Am J Psychiatry 158(8): 1206-1214.

Seto, S. W., G. Y. Yang, H. Kiat, A. Bensoussan, Y. W. Kwan and D. Chang (2015). "Diabetes Mellitus, Cognitive Impairment, and Traditional Chinese Medicine." Int J Endocrinol 2015: 14.

Shoji-Kasai, Y., H. Ageta, Y. Hasegawa, K. Tsuchida, H. Sugino and K. Inokuchi (2007). "Activin increases the number of synaptic contacts and the length of dendritic spine necks by modulating spinal actin dynamics." Journal of Cell Science 120(Pt 21): 3830-3837.

Shukitt-Hale, B., V. Cheng and J. A. Joseph (2009). "Effects of blackberries on motor and cognitive function in aged rats." Nutr Neurosci 12(3): 135-140.

Solfrizzi, V. and F. Panza (2014). "Mediterranean diet and cognitive decline. A lesson from the whole-diet approach: what challenges lie ahead?" J Alzheimers Dis 39(2): 283-286.

Spencer, J. P. (2009). "Flavonoids and brain health: multiple effects underpinned by common mechanisms." Genes Nutr 4(4): 243-250.

Spencer, J. P., K. Vafeiadou, R. J. Williams and D. Vauzour (2012). "Neuroinflammation: modulation by flavonoids and mechanisms of action." Mol Aspects Med 33(1): 83-97.

Stangl, D. and S. Thuret (2009). "Impact of diet on adult hippocampal neurogenesis." Genes Nutr 4(4): 271-282.

Suganuma, M., S. Okabe, M. Oniyama, Y. Tada, H. Ito and H. Fujiki (1998). "Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue." Carcinogenesis 19(10): 1771-1776.

Sullivan, E. L., E. K. Nousen and K. A. Chamlou (2014). "Maternal high fat diet consumption during the perinatal period programs offspring behavior." Physiol Behav 123: 236-242.

Sunram-Lea, S. I., J. K. Foster, P. Durlach and C. Perez (2001). "Glucose facilitation of cognitive performance in healthy young adults: examination of the influence of fast-duration, time of day and pre-consumption plasma glucose levels." Psychopharmacology (Berl) 157(1): 46-54.

Talavéra, S., C. Felgines, O. Texier, C. Besson, A. Gil-Izquierdo, J.-L. Lamaison and C. Rémésy (2005). "Anthocyanin Metabolism in Rats and Their Distribution to Digestive Area, Kidney, and Brain." J Agric Food Chem 53(10): 3902-3908.

Tsai, T. H. and Y. F. Chen (2000). "Determination of unbound hesperetin in rat blood and brain by microdialysis coupled to microbore liquid chromatography." J Food Drug Analys 8(4): 331-336.

van der Zwaluw, N. L., O. van de Rest, R. P. C. Kessels and L. C. P. G. M. de Groot (2015). Effects of glucose load on cognitive functions in elderly people.

Vaughan, R. A. and J. D. Foster (2013). "Mechanisms of dopamine transporter regulation in normal and disease states." Trends Pharmacol Sci 34(9): 489-496.

Vauzour, D. (2012). "Dietary polyphenols as modulators of brain functions: biological actions and molecular mechanisms underpinning their beneficial effects." Oxid Med Cell Longev 2012: 914273.

Vauzour, D., K. Vafeiadou, A. Rodriguez-Mateos, C. Rendeiro and J. P. Spencer (2008). "The neuroprotective potential of flavonoids: a multiplicity of effects." Genes Nutr 3(3-4): 115-126.

Visioli, F., C. A. De La Lastra, C. Andres-Lacueva, M. Aviram, C. Calhau, A. Cassano, M. D'Archivio, A. Faria, G. Fave, V. Fogliano, R. Llorach, P. Vitaglione, M. Zoratti and M. Edeas (2011). "Polyphenols and human health: a prospectus." Crit Rev Food Sci Nutr 51(6): 524-546.

Vogiatzoglou, A., A. A. Mulligan, M. A. Lentjes, R. N. Luben, J. P. Spencer, H. Schroeter, K. T. Khaw and G. G. Kuhnle (2015). "Flavonoid intake in European adults (18 to 64 years)." PLoS One 10(5): e0128132.

Walle, T. (2004). "Absorption and metabolism of flavonoids." Free Radic Biol Med 36(7): 829-837.

Wang, X., Y. Y. Ouyang, J. Liu and G. Zhao (2014). "Flavonoid intake and risk of CVD: a systematic review and meta-analysis of prospective cohort studies." Br J Nutr 111(1): 1-11.

Williams, R. J. and J. P. Spencer (2012). "Flavonoids, cognition, and dementia: actions, mechanisms, and potential therapeutic utility for Alzheimer disease." Free Radic Biol Med 52(1): 35-45.

Williams, R. J., J. P. Spencer and C. Rice-Evans (2004). "Flavonoids: antioxidants or signalling molecules?" Free Radic Biol Med 36(7): 838-849.

Williamson, G. (2013). "Possible effects of dietary polyphenols on sugar absorption and digestion." Mol Nutr Food Res 57(1): 48-57.

Williamson, G. and B. Holst (2008). "Dietary reference intake (DRI) value for dietary polyphenols: are we heading in the right direction?" Br J Nutr 99 Suppl 3: S55-58.

Williamson, G. and C. Manach (2005). "Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies." Am J Clin Nutr 81(1 Suppl): 243S-255S.

Willis, L. M., B. Shukitt-Hale and J. A. Joseph (2009). "Recent advances in berry supplementation and age-related cognitive decline." Curr Opin Clin Nutr Metab Care 12(1): 91-94.

Wolf, Y., S. Yona, K. W. Kim and S. Jung (2013). "Microglia, seen from the CX3CR1 angle." Front Cell Neurosci 7: 26.

Yang, Y., L. Bai, X. Li, J. Xiong, P. Xu, C. Guo and M. Xue (2014). "Transport of active flavonoids, based on cytotoxicity and lipophilicity: An evaluation using the blood–brain barrier cell and Caco-2 cell models." Toxicology in Vitro 28(3): 388-396.

Yirmiya, R., N. Rimmerman and R. Reshef (2015). "Depression as a Microglial Disease." Trends in Neurosci 38(10): 637-658.

Youdim, K. A., M. S. Dobbie, G. Kuhnle, A. R. Proteggente, N. J. Abbott and C. Rice-Evans (2003). "Interaction between flavonoids and the blood-brain barrier: in vitro studies." J Neurochem 85(1): 180-192.

Youdim, K. A., J. P. Spencer, H. Schroeter and C. Rice-Evans (2002). "Dietary flavonoids as potential neuroprotectants." Biol Chem 383(3-4): 503-519.

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but it is the journey that matters, in the end."

Ernest Hemingway

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